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DOUTORAMENTO

CIÊNCIAS BIOMÉDICAS

Hormonal regulation of spermatogenesis: role of ghrelin-leptin axis and glucagon-like peptide 1 (GLP1) in nutrient membrane transport through Sertoli cell/ blood-testis barrier

Ana Catarina Dias Martins

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Tese de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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“The development of science is not a direct march to the truth. If not for false starts and blind alleys, scientists would be traveling for too long down too many wrong paths.”

Brilliant Blunders
From Darwin To Einstein
From: Mario Livio

Declaração de Honra

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(Ana Catarina Dias Martins)

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List of Publications

Publications included in this thesis:

1. **Martins AD**, Moreira AC, Sá R, Monteiro MP, Sousa M, Carvalho RA, Silva BM, Oliveira PF, Alves MG (2015). Leptin modulates human Sertoli cells acetate production and glycolytic profile: a novel mechanism of obesity-induced male infertility? Biochimica et Biophysica Acta - Molecular Basis of Disease. 1852(9): 1824-1832 (DOI: 10.1016/j.bbadis.2015.06.005)

2. **Martins AD,** Sá R, Monteiro MP, Barros A, Carvalho RA, Silva BM, Oliveira, PF, Alves MG (2016). Ghrelin acts as energy status sensor of male reproduction by modulating Sertoli cells glycolytic metabolism and mitochondrial bioenergetics. Molecular and Cellular Endocrinology. 434:199-209 (DOI: 10.1016/j.mce.2016.07.008)

3. Meneses MJ, **Martins AD** (2017) Hormonal Control of Male Reproductive Function. In: Alves MG, Oliveira PF (eds) Biochemistry of Andrology, vol 1. Andrology: Current and Future Developments. Bentham Science Publishers, Sharjah, UAE, pp 126-153. ISBN: 978-1-68108-501-2 (Book Chapter)

4. **Martins AD**, Monteiro MP, Silva BM, Barros A, Sousa M, Carvalho RA, Oliveira PF, Alves MG (2019) Metabolic dynamics of human Sertoli cells are differentially modulated by physiological and pharmacological concentrations of GLP-1. Toxicology and Applied Pharmacology. 362:1-8 (DOI: 10.1016/j.taap.2018.10.009)

5. **Martins AD**, Oliveira PF, Alves MG (2019) Assessment of Sertoli cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and sulforhodamine B assays. Current Protocols in Toxicology. 81(9):e81 *(*DOI: 10.1002/cptx.85)

6. **Martins AD**, Jarak I, Morais T, Carvalho RA, Oliveira PF, Monteiro MP, Alves MG (2019) Caloric restriction alters the hormonal profile and testicular metabolome resulting in alterations of sperm morphology - Submitted

Abstract

The changes in food habits and lifestyle have consequences to human health including obesity, one of the most alarming diseases in modern societies. Indeed, obesity rates have been increasing in the last decades, and currently is not only a problem in modern societies, but also in countries under development. In Portugal is estimated that 53% of the population above 18 years old is overweight or obese. Reports have associated the increase in the incidence of metabolic disorders, particularly obesity, with the decrease in fertility rates that we are witnessing. Indeed, in males, the increase in subfertility/infertility rates may be due to several factors. Although it is known that hormones tightly regulate spermatogenesis, the effect of hormones known to be dysregulated by metabolic diseases (for example: leptin, ghrelin) in Sertoli cells and spermatogenesis remained to be elucidated. In addition, there is an increase in therapies directed to obesity, though the effects of those therapies in spermatogenesis also remain unclear and overlooked. Bariatric surgery and imposition of caloric restriction have many effects in the metabolic and hormonal profiles of males. While consequences of caloric restriction in the female reproductive system are well known, the effects to male reproductive potential have been neglected.

Sertoli cells are the functional units inside the testis and the main responsible for the success of spermatogenesis. They are responsible for the structural support of the developing germ cells, for the establishment of a local immune privileged environment, and for the nutritional support of germ cells. The substrates and factors secreted by Sertoli cells, such as lactate, are extremely relevant for the development of fully capable spermatozoa. Thus, in the first sections of this work we studied the effects of leptin and ghrelin in the metabolism of *in vitro* cultured human Sertoli cells. The following section aimed to explore the effects of glucagon-like peptide-1 (GLP-1) to the nutritional support of spermatogenesis. Finally, we also evaluated the *in vivo* effects of a caloric restriction, associated with dysregulation in these hormones, in testicular metabolic profile and sperm quality.

To achieve those objectives, firstly human Sertoli cells were cultured with increasing concentrations of leptin (5 ng/mL according the physiological levels found in lean, healthy patients and the concentration found in seminal plasma; 25 ng/mL based on the levels reported for obese patients; and 50 ng/mL based on the levels reported in morbidly obese men) or ghrelin (20 pM was selected based on the levels reported in obese; the concentration of 100 pM was selected based on the levels measured in normal weight individuals; and the concentration of 500 pM was chosen based on the levels of ghrelin reported in severely undernourished individuals). GLP-1 is

another hormone associated with obesity and food balance. Thus, we also evaluated the effect of increasing concentrations of GLP-1 (0.01 nM of GLP-1 to mimic physiological post-prandial levels; or to pharmacological levels used for obesity treatment, 1 and 100 nM) on human Sertoli cells.

The mRNA expression of the receptor for each hormone was firstly confirmed by PCR in human Sertoli cells exposed to leptin, ghrelin or GLP-1. Moreover, our data showed that GLP-1 receptor expression in human Sertoli cells was not affected by exposure to GLP-1, while ghrelin receptor expression increased after an exposure to 100 pM of ghrelin in human Sertoli cells. The consumption/production of extracellular metabolites in the cells of those experimental groups was assessed by ^{1}H-NMR, while protein levels and/or activities of glycolysis-related enzymes and transporters were assessed by Western Blot and commercial kits, respectively. The bioenergetic profile of the cells was evaluated through the study of the protein levels of mitochondrial complexes and/or mitochondrial membrane potential. Finally, oxidative damages were evaluated thought the quantification of protein levels of oxidative stress markers (carbonyl groups, nitro tyrosine and 4-Hydroxynonenal) by slot blot.

Our data showed that all concentrations of leptin decreased acetate production by human Sertoli cells, and did not induce or protected those cells against oxidative stress. Regarding ghrelin, there was an inverse association between exposure to increasing levels of ghrelin and lactate production. Additionally, human Sertoli cells exposed to GLP-1 showed an increase in the efficiency of lactate production, without the occurrence of oxidative damages or toxicity. These results suggest that ghrelin acts as an energy sensor and GLP-1 can counteract the negative effects of obesity in male reproductive function. Taken together, these results showed that leptin, ghrelin and GLP-1 impact the nutritional support of the spermatogenesis.

To further characterize the effects of hormonal dysfunction caused by food intake, we studied the effect of caloric restriction in male fertility. Male Wistar rats were divided into a group fed with 30% less calories (subjected to caloric restriction) than weight matched *ad libitum* fed animals (control group), for 28 days. The hormonal profile (leptin, ghrelin and GLP-1) was assessed using commercial kits. The levels of receptors for those hormones were analyzed by qPCR. Sperm parameters were also evaluated. The metabolic profile of the testis was determined through ^{1}H-NMR based metabolomics. The expression of glycolysis-related transporters and enzymes was assessed by Western blot. The expression of testicular mitochondrial complexes and biogenesis were analyzed. Finally, testicular tissue and spermatozoa were tested for oxidative stress markers. The animals subjected to caloric restriction presented lower body weight, as well as an altered hormonal profile. Although no significant changes were found in the testicular content of

the metabolites analyzed there was a clear separation of the metabolic profile between the two experimental groups. In rats subjected to caloric restriction, a decrease in oxidative stress markers in testicular tissue and spermatozoa was observed. Still, there was an increase in sperm head defects in spermatozoa from rats subjected to caloric restriction. Our results highlight that caloric restriction affects testicular energetic homeostasis with implications to male fertility. Further studies will be needed to unveil the routes of action for each of these hormones in the male reproductive system.

In conclusion, these works elucidate some of the mechanisms by which leptin, ghrelin and GLP-1 alter the nutritional support of spermatogenesis by Sertoli cell and thus, male fertility. In addition, our data highlights that food intake, which alters the hormonal balance of ghrelin, leptin and GLP-1, affects sperm quality. Particularly, we report negative effects of caloric restriction to testicular metabolism and sperm quality. Overall, we conclude that these hormones are pivotal for testicular energy balance particularly towards sustaining the nutritional support of spermatogenesis in a controlled manner.

Resumo

Maus hábitos alimentares e um estilo de vida sedentário têm consequências que podem ser nefastas para a saúde humana, incluindo o aumento de peso ou mesmo obesidade. A obesidade é atualmente uma das doenças mais alarmantes nas sociedades modernas. O aumento da prevalência de obesidade a nível mundial nas últimas décadas indica que este problema já não é apenas exclusivo das sociedades desenvolvidas, mas também motivo de preocupação nos países em desenvolvimento. Em Portugal, estima-se que cerca de 53% da população adulta (com 18 ou mais anos) tem excesso de peso ou é obesa. De particular importância são os estudos recentes que relacionam o aumento da prevalência de doenças metabólicas, particularmente a obesidade, com uma diminuição da taxa de fertilidade. No caso masculino, o aumento da subfertilidade e/ou infertilidade deve-se a vários fatores. A regulação hormonal da espermatogénese é extremamente importante e as doenças metabólicas são caracterizadas e consequência de flutuações hormonais. No entanto, o efeito da desregulação hormonal nas células de Sertoli e, consequentemente, na espermatogénese ainda carece de esclarecimento. Como consequência do aumento da prevalência de obesidade verifica-se um aumento da comercialização de fármacos prescritos no sentido de diminuir o peso, no entanto os seus efeitos na espermatogénese também permanecem desconhecidos. Existe ainda o recurso à cirurgia bariátrica e a imposição de uma restrição calórica com a finalidade de perda de peso, sendo que ambos têm efeitos no metabolismo e perfil hormonal. Enquanto as consequências da restrição calórica no sistema reprodutor feminino estão bem estudadas, os efeitos no potencial reprodutivo masculino são negligenciados.

As células de Sertoli são as unidades funcionais do testículo e as responsáveis pelo sucesso da espermatogénese sendo que fazem o suporte estrutural e nutricional para as células germinativas em desenvolvimento e criam um microambiente de privilégio imunológico. Os substratos e os outros fatores libertados pelas células de Sertoli, como o lactato, são extremamente importantes para o correto desenvolvimento dos espermatozoides. Deste modo, na primeira parte deste trabalho, estudamos o efeito da leptina e da grelina no metabolismo de células de Sertoli humanas em cultura. Depois exploramos os efeitos do péptido semelhante à glicagina-1 (GLP-1) no suporte nutricional da espermatogénese. Por fim, estudamos os efeitos de uma restrição calórica, associada à desregulação hormonal, no metabolismo testicular e na qualidade espermática.

Para atingir os objetivos definidos, primeiramente células de Sertoli humanas foram cultivadas com concentrações crescentes de leptina (5 ng/mL correspondente a níveis fisiológicos

presentes em homens saudáveis com peso normal e também à concentração encontrada no plasma seminal; 25 ng/mL baseado nos níveis encontrados em pacientes obesos; e 50 ng/mL baseado nos níveis reportados em homens com obesidade mórbida) ou grelina (20 pM selecionada de acordo com os níveis reportados em homens obesos; a concentração de 100 pM selecionada com base nos níveis registados em indivíduos com peso normal; e a concentração de 500 pM escolhida com base nos níveis de grelina reportados em indivíduos severamente desnutridos). O GLP-1 é outra hormona também associada à obesidade e ao balanço energético. Assim, nós avaliamos o efeito de concentrações crescentes de GLP-1 (0.01 nM de GLP-1 de forma a mimetizar os níveis pós-prandiais; ou os níveis farmacológicos usados no tratamento da obesidade, 1 e 100 nM) em células de Sertoli humanas.

A expressão de mRNA para o recetor de cada hormona foi primeiramente confirmada por PCR em células de Sertoli humanas expostas à leptina, grelina ou GLP-1. Adicionalmente, os nossos resultados demonstram que a expressão do recetor de GLP-1 em células de Sertoli humanas não foi afetado pela exposição ao GLP-1, enquanto o recetor da grelina aumentou a sua expressão em células de Sertoli humanas após exposição a 100 pM de grelina. O consumo/produção de metabolitos extracelulares nestes grupos foi medido por ^{1}H-RMN enquanto os níveis de proteína e/ ou atividades de enzimas e transportadores importantes na glicólise foram medidos por Western Blot e recorrendo a kits comerciais, respetivamente. O perfil bioenergético das células foi avaliado estudando os níveis de proteína dos complexos mitocondriais e/ou o potencial membranar mitocondrial. Por fim, por Slot Blot, foram avaliados os danos oxidativos recorrendo aos níveis de proteína de marcadores de stress oxidativo (grupos carbonilo, nitrotirosina e 4-hidroxinonenal).

Os resultados obtidos mostram uma diminuição na produção de acetato pelas células de Sertoli quando expostas a leptina. Para além disso, o tratamento com leptina não induz nem protege as células de Sertoli contra o stress oxidativo. Em células de Sertoli expostas a grelina, verifica-se uma associação inversa entre o aumento dos níveis de grelina e a produção de lactato. Adicionalmente, células de Sertoli humanas expostas a GLP-1 demonstram uma eficiência na produção de lactato sem a presença de danos oxidativos ou toxicidade. Deste modo, é sugerido que a grelina funciona como um sensor metabólico energético e os efeitos do GLP-1 podem contrabalançar os efeitos negativos da obesidade na função reprodutiva do homem. De um modo geral, os resultados mostram que a leptina, a grelina e o GLP-1 afetam o suporte nutricional da espermatogénese.

Para um estudo mais completo sobre os efeitos da disfunção hormonal como consequência do regime alimentar, incluímos, neste trabalho, o estudo dos efeitos da restrição calórica na

fertilidade masculina. Ratos Wistar machos foram divididos aleatoriamente em dois grupos: um grupo alimentado com menos 30% de calorias (sujeito a uma restrição calórica) que o outro grupo alimentado *ad libitum* (grupo controlo) durante 28 dias. O perfil hormonal (leptina, grelina e GLP-1) foi avaliado através de kits comerciais. Os níveis de recetores para estas hormonas foram analisados por qPCR. Parâmetros espermáticos foram também avaliados. O perfil metabólico testicular foi determinado por ^{1}H-RMN. A expressão de transportadores e enzimas importantes na glicólise foi analisada por Western Blot. A expressão dos complexos mitocondriais do tecido testicular e a biogénese foi analisada. Por fim, no tecido testicular e espermatozóides foram medidos marcadores de stress oxidativo. Os animais sujeitos à restrição calórica apresentaram menor peso, assim com um perfil hormonal alterado. Apesar dos níveis de metabolitos analisados no tecido testicular não apresentarem diferenças, foi observada uma clara separação do perfil metabólico entre os dois grupos experimentais. Nos ratos sujeitos à restrição calórica observámos uma diminuição nos marcadores de stress oxidativo no tecido testicular e nos espermatozóides. Contudo, foi detetado um aumento nos defeitos de cabeça dos espermatozóides de ratos sujeitos à restrição calórica. Os resultados obtidos provam que a restrição calórica afeta a homeostase energética no testículo com implicação na fertilidade masculina. No entanto, mais estudos são necessários para elucidar completamente os mecanismos e vias de ação destas hormonas no sistema reprodutor masculino.

Em conclusão, estes trabalhos permitem elucidar alguns dos mecanismos pelos quais a leptina, a grelina e o GLP-1 afetam o suporte nutricional pelas células de Sertoli e consequentemente a fertilidade masculina. Os resultados obtidos destacam também que a modulação do balanço hormonal da leptina, grelina e GLP-1 resultante do regime alimentar afeta a qualidade espermática. Os resultados obtidos revelam ainda efeitos negativos da restrição calórica no metabolismo testicular e qualidade espermática. Concluímos assim que as hormonas em estudo (leptina, grelina e GLP-1) são importantes no balanço energético testicular, promovendo um maior controlo sobre o suporte nutricional da espermatogénese.

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List of Abbreviations

4-HNE - 4-Hydroxynonenal

^{1}H-NMR - Proton Nuclear Magnetic Resonance

AgRP - Agouti-related protein

Ala - Alanine

Asp - Aspartic acid

AUA - American Urological Association

BCAA - Branched-chain amino acids

BMI - Body mass index

BTB - Sertoli-Sertoli cell barrier/ Blood testis barrier

CBAVD - Congenital bilateral absence of the vas deferens

cDNA - Complementary DNA

CFTR - Cystic fibrosis transmembrane conductance regulator

CR - Caloric restriction

DBD - DNA binding domain

DHT - 5α-dihydrotestosterone

DNP - Dinitrophenyl

EAA - European Academy of Andrology

ERKs - Extracellular signal-regulated kinases

ERs - Estrogen receptors

ESHRE - European Society of Reproductive Medicine and Embryology

FBS - Fetal bovine serum

FDA - Food and Drug Administration

FSH - Follicle-stimulating hormone

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase;

GH - Growth hormone

GHR - Growth hormone receptor

GHS-R - Growth hormone secretagogue receptor

GLP-1 - Glucagon-like peptide 1

GLP-1R - Glucagon like peptide-1 receptor

Glu - Glutamate

GLUTs - Glucose transporters

GnRH - Gonadotropin releasing hormone

GnRHR - GnRH cell membrane receptors

GPER - G-protein-coupled estrogen receptor 1

GPT - Glutamate pyruvate transaminase

HADHB - Mitochondrial trifunctional protein subunit beta

$HBSS_f$ - Hanks Balanced Salt Solution without Ca2+ or Mg2+

HPG - Hypothalamic-pituitary gonadal

IGF-1 - Insulin-like growth factor 1

ITS - Insulin–transferrin–sodium selenite

JC-1 - Cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide

KS - Klinefelter syndrome

LBD - C-terminal ligand binding domain

LDH - Lactate dehydrogenase

LepR or OB-R – Leptin or Obesity receptor

LH - Luteinizing hormone

MCTs - Monocarboxylate transporters

mTOR - Mammalian target of rapamycin

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

ND1 - NADH-ubiquinone oxidoreductase chain 1

NRF1 - Nuclear respiratory factor 1

NRs - Nuclear receptors

NT - Nitro-tyrosine

NTD - N-terminal domain

OD - Optic density

OS - Oxidative stress

P450arom - Cytochrome P450 aromatase

PDH -Pyruvate dehydrogenase;

PFK- Phosphofructokinase

PGC-1α - Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI3K - Phosphatidylinositol 3-kinase

PKB-P - Phosphorylated protein kinase B

PKM1/2 - Pyruvate kinase M1/2 isoform;

POMC - Neurons producing anorexigenic proopiomelanocortin

PPARγ - Peroxisome proliferator-activated receptor gamma

Pro - Proline

qPCR - Quantitative real-time PCR

ROS - Reactive oxygen species

RT- PCR - Reverse transcriptase polymerase chain reaction

Sirt1 - Sirtuin 1

SPM - Sperm preparation medium

SRB - Sulforhodamine B

StAR - Steroidogenic acute regulatory protein

T3 - 3,30,5-triiodothyronine

T4 - 3,3’,5,5’-tetraiodothyronine

TBS - Tris-buffered saline solution

TESE - Testicular sperm extraction

TH - Thyroid hormones

THR - Thyroid hormone receptor

TOCSY - Total correlation spectroscopy

tRNA - total RNA

TSH - Thyrotropin/thyroid-stimulating hormone

UCP2 - Uncoupling protein 2

WHO - World Health Organization

XTT - Sodium 3′-[1-[(phenylamino)-carbony]-3, 4-tetrazolium]-bis (4- methoxy-6-nitro) benzene-sulfonic acid hydrate

β2M - β-2-microglobulin

$\beta 2M_{nc}$ - Nuclear encoded beta-2-microglobulin

Chapter 1

Introduction

Introduction

Infertility

Infertility was classified as a disease not many years ago and prevails worldwide being a life quality issue, although it does not represent a direct risk to health. According to the World Health Organization (WHO), infertility is a disease of the reproductive system clinically defined as the incapacity to generate and maintain a full-term pregnancy after at least 12 months of unprotected and regular sexual intercourse [1]. The prevalence of infertility can vary between regions but is increasing worldwide [2-5]. It is estimated that one in six couples will suffer from fertility issues [6]. According to the European Society of Reproductive Medicine and Embryology (ESHRE), infertility can be attributed to different causes, but male and female problems share relevant contribution. Between 20-30% of the causes are attributed to men, 20-35% to the female while 25-40% is attributed to both and in approximately 10-20% of the cases the cause is unknown [6]. The possible causes for male infertility are extensive and can have a genetic origin; be a consequence of a medical condition or disease; and more recently has been highlighted the possible effects of lifestyle.

Regarding genetic causes, Klinefelter syndrome (KS) is the most common cause of azoospermia [7-9], together with chromosomal translocations [7, 9]. Congenital bilateral absence of the vas deferens (CBAVD) is a genetic disorder that can be identified by a physical exam and confirmed by genetic test [7, 10]. In this condition, the majority of the patients have mutated versions of the cystic fibrosis transmembrane conductance regulator (CFTR) gene [7, 11, 12]. In fact, the American Urological Association (AUA) and the European Academy of Andrology (EAA) guidelines recommend the karyotype test for all men with non-obstructive azoospermia and a total motile count of less than 5 million of spermatozoa [13].

Other causes for male infertility are related with inflammatory reactions in the reproductive tract and represent about 10% of infertility cases [14]. For instance, *Chlamydia trachomatis* is responsible for urethritis, epididymitis and prostatitis [15]. The current knowledge on the effects of this infection on male fertility was mainly raised from studies in animal models and *in vitro* experiments (reviewed by [15]). There are many other pathogens, including *Neisseria gonorrhea, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* among other that affect male reproductive tract [16].

There are also systemic diseases that can cause male subfertility/infertility, including non-controlled [17, 18] or undiagnosed diabetes [18, 19], vitamin D insufficiency [20] or autoimmune diseases [21]. Obesity is other systemic disease that can affect fertility. The effects of this non-communicable disease on male reproductive tract are extensively described in the literature (for review [22]). In brief, obesity can affect fertility through hormonal dysregulation, sperm DNA fragmentation, erectile dysfunction, hyperthermia and epigenetic changes [23] (Figure 1).

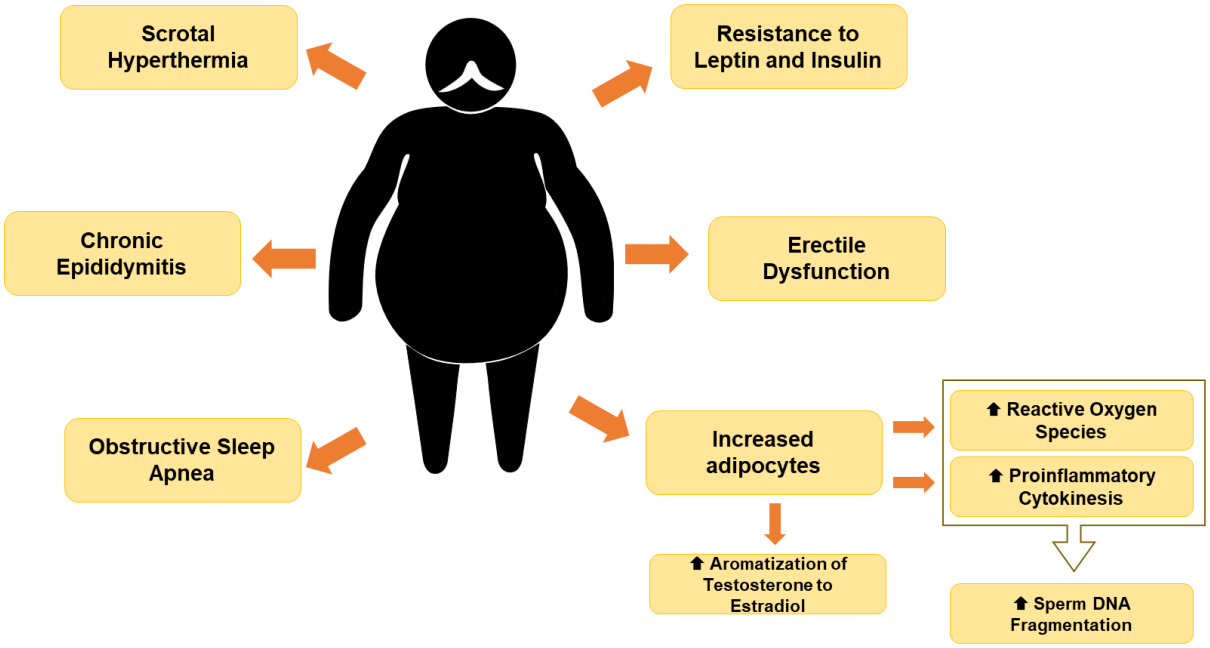

Figure 1. Summary of the main mechanisms by which obesity causes male subfertility/infertility.

Hormonal homeostasis is essential to spermatogenesis regulation and consequently for its success. Obesity is associated with a strong dysregulation of the endocrine system. For instance, the aromatization of testosterone to estrogen that occurs in overweight and obese men affects the hypothalamus-pituitary-gonad (HPG) axis. Furthermore, leptin, ghrelin and glucagon like peptide-1 are also dysregulated and since testicular cells are hormonal targets, they can have direct effects on testicular metabolic homeostasis, as discussed in detail in chapter 2.

Lifestyle of the individuals has been associated with their fertility [24, 25] though those effects can be voluntary or involuntary. While involuntary environmental factors include pesticides, which are widely studied [26-32], other factors such as alcohol consumption or smoking are considered voluntary environmental factors of exposure. For example, smoking and alcohol consumption increase sperm DNA damage [33] and reduce sperm quality [34, 35]. Nowadays, modern societies impose great pressure in professional and personal life of men that when under

psychological stress suffer with fertility problems [36-38]. More recently, the increasing concern with appearance and weight are gaining relevance as factors that promote stress pressure in males, thus also affecting their fertility. The search for an ideal appearance through exercise or diet is a current trend. The effects of excess of exercise remain largely unknown but it is suggested that it may negatively affect fertility as supported by studies done in professional athletes [39-42]. On the other hand, the effects of dietary habits on male fertility are widely studied, particularly the effects of dietary patterns [43-45] or supplements and vitamins [46, 47]. In addition, voluntary or imposed caloric restriction is also known to induce some deleterious effects on sperm quality or testicular function, though the available data was mainly obtained from animal models [48, 49]. However, the beneficial effects of an equilibrated diet and moderate exercise to male fertility must be taken into consideration [42, 50]. The fertility status of the individual is primarily evaluated by studying sperm quality [51]. However, sperm quality and quantity only reflect the endpoint of spermatogenesis without taking in consideration that spermatozoa are produced inside the seminiferous tubules [52] and result from a strict cooperation between germ and Sertoli cells. Studying this later process is also necessary to determine the quality of the reproductive process in males.

Morphological and functional aspects of Sertoli Cells

Sertoli cells have a polygonal shape and a nuclei with an irregular shape [53]. From the basement membrane to the lumen of the seminiferous tubules, Sertoli cells present a columnar shape, which allows them to contact with the surface of multiple developing germ cells [54] (Figure 2). Indeed, the amount of developing germ cells supported during spermatogenesis is associated with the number of functional Sertoli cells [55]. Sertoli cells are the main structural elements of the seminiferous tubules being responsible for the developing germ cells and thus, spermatogenesis [56]. The functions of Sertoli cells include nutritional support of the developing germ cells; modulation of apoptosis; posterior phagocytosis of the apoptotic germ cells; incorporation of hormonal signals and endocrine signals; and secretion of hormones and factors crucial for the developing germ cells [56-59]. The composition of the tubular fluid is also strictly controlled by Sertoli cells. In addition, they create a local immune-privileged environment inside the seminiferous tubules due to the formation of the blood-testis barrier [60]. This barrier is formed by adjacent Sertoli cells interjoined by tight junctions, basal ectoplasmic specializations, basal tubulobulbar complexes, desmosome-like junctions and gap junctions established alongside in the seminiferous epithelium [61]. The blood-testis barrier (BTB) is responsible for the selection of molecules that enter in the tubular lumen from the interstitial fluids [60]. The germ cells at certain

point express auto antigens and the BTB creates a protected environment, protecting these cells from the immune system [62].

Spermatogenesis consists of a series of cellular events that begin with mitosis of spermatogonia, where one remains to renewal the pool of these cells and other spermatogonia continues the differentiation, outside the BTB, in the basal compartment, to type B spermatogonia and then to primary spermatocytes. Primary spermatocytes are the cells in transit thought the BTB [60, 63, 64]. After the BTB, the cell progresses after the first meiosis to secondary spermatocytes and after second meiosis to round spermatids, and then in elongated spermatids. The spermatozoa are formed next and released in the lumen of the seminiferous tubules through spermiation [60, 65] (Figure 2).

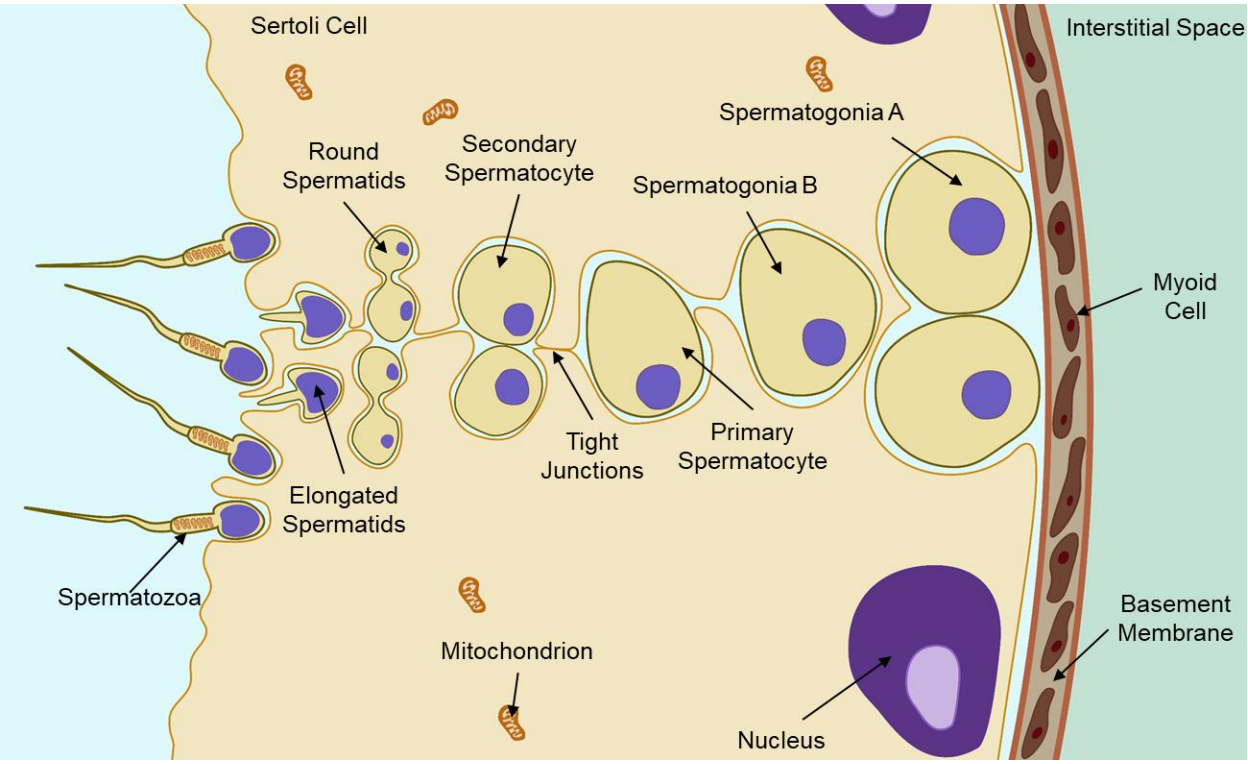

Figure 2. Schematic representation of the seminiferous tubules. The Sertoli cell barrier physically splits the seminiferous epithelium into basal and apical (adluminal) compartments. Tight junctions are established between adjacent Sertoli cells forming the Sertoli/blood–testis barrier. The germ cells are in intimate association with Sertoli cells, and each cell supports multiple germ cells. The Sertoli cells are responsible for physical and nutritional support of germ cells. These cells also control the access of substances from the intratubular fluid and secrete several factors essential for germ-cell development. In addition, Sertoli cell creates a microenvironment where germ cells mature into completely mature spermatozoa.

Metabolic cooperation between germ cells and Sertoli cells

The nutritional support of the developing germ cells is assured by Sertoli cells. Without the energy substrates provided by Sertoli cells, the germ cells degenerate and are eliminated [66]. The energetic needs of the germ cells vary along the development from spermatogonia to spermatozoa [67]. However, pyruvate and lactate produced by Sertoli cells, through metabolization of glucose and other substrates, are the preferential substrates used by the developing germ cells [68, 69].

Glucose is transported to the Sertoli cells across the cell membrane through glucose transporters (GLUTs) (Figure 3). These proteins mediate the passive transport of glucose through the phospholipidic bilayer [70]. The GLUT family has several isoforms [70] being that in Sertoli cells have been identified GLUT1 [71-77], GLUT2 [73, 75, 76, 78, 79], GLUT3 [72-77, 80] and GLUT8 [81]. Among the identified isoforms, glucose transport to inside the Sertoli cell by GLUT8 is excluded since this isoform is not localized in the plasmatic membrane and its localization is still controversial [82]. GLUT1 [83], GLUT2 [84] and GLUT3 [83, 84] are well identified in the plasma membrane of the Sertoli cells and thus, their role in glucose transport is evident. Once inside the Sertoli cell, glucose is metabolized through glycolysis, although with a lower efficiency in ATP generation. Nevertheless, this process is preferred by these cells instead of mitochondrial phosphorylation [85]. However, the lower efficiency of ATP production is overcome by the high glycolytic flux known to occur in Sertoli cells [85]. The modulation of GLUTs expression, particularly by hormones and associated factors, is reported to have a role in maintaining a correct functioning of the Sertoli cells [86, 87]. Once inside the cell, glucose is converted to pyruvate through glycolysis. Among the many enzymes involved in glycolysis, phosphofructokinase is considered the first limiting step of this process [88]. The pyruvate obtained at the end of glycolysis can follow different destinations: be converted to alanine by the action of alanine aminotransferase (ALT) or enter the mitochondria to be incorporated in the Krebs cycle [85, 89]. The action of lactate dehydrogenase (LDH) that converts pyruvate to lactate and reversibly the latter to the former, is accompanied by the conversion of $NADH/H^+$ to NAD^+ [90]. The production of lactate by Sertoli cells is modulated by endocrine factors [66, 73, 75, 91]. After being produced lactate is exported to the extracellular space to be incorporated by germ cells, constituting their preferential substrate [69]. The transport of lactate occurs through monocarboxylate transporters (MCTs) [92]. In Sertoli cells, the transporter responsible for lactate import from circulation is the MCT4 [77, 87] while the one responsible for its export to the intratubular space where it will be used by developing germ cells is MCT2 [93, 94] (Figure 3). Sertoli cells metabolism is mainly directed to glycolysis and glutaminolysis, being the same observed in cancer cells metabolism.

However, in Sertoli cells this mainly occurs to produce metabolic substrates for developing germ cells, whereas in cancer cells the metabolites produced by this pathway are mainly used for cell proliferation [85, 95, 96].

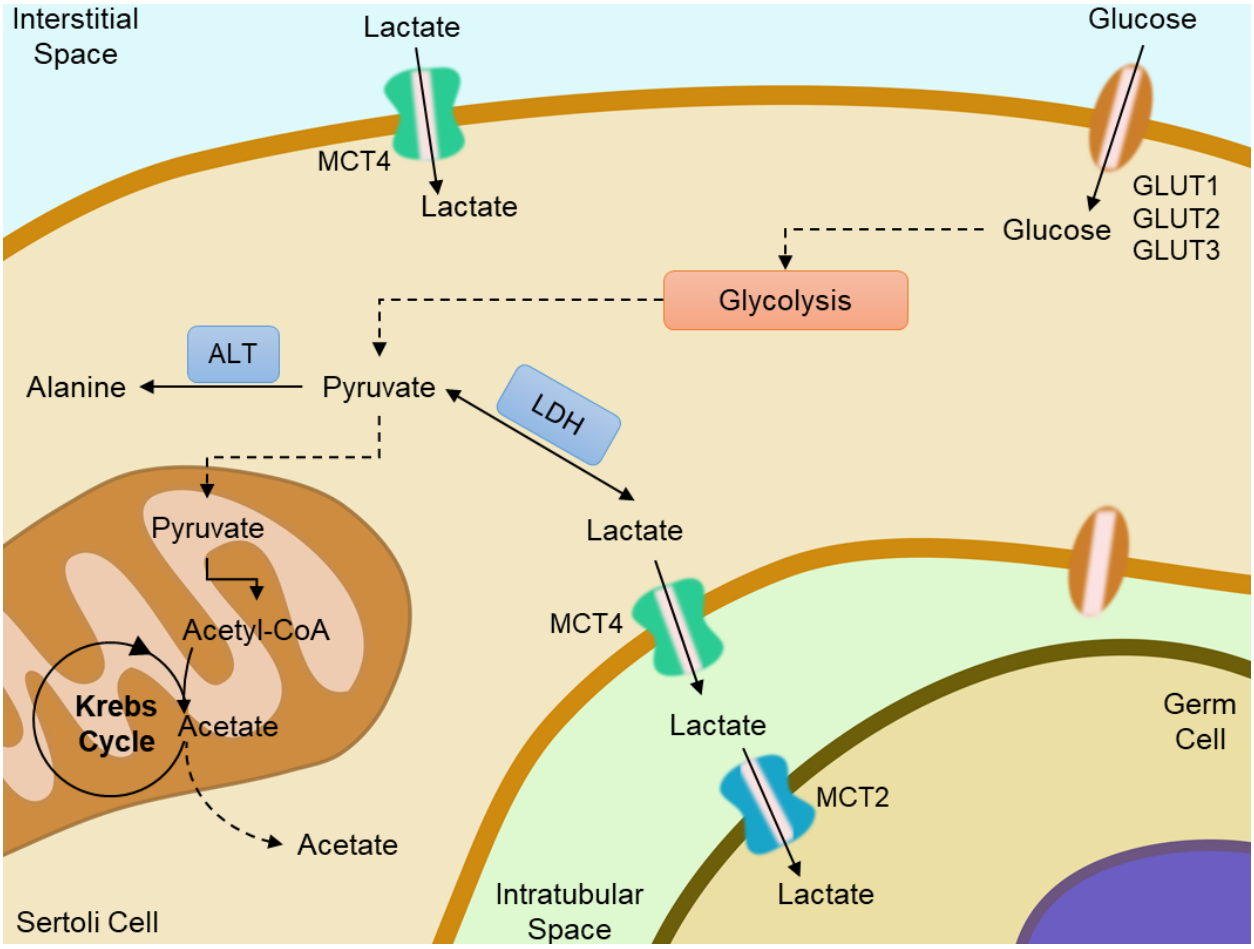

Figure 3. Schematic illustration of Sertoli cell metabolism. Glucose is the preferred metabolite used by Sertoli cells and is imported from circulation by glucose transporters (GLUTs). Most of the glucose is metabolized through glycolysis to pyruvate. The resulting pyruvate can be then converted into lactate, or alanine, or be transported to the mitochondrial matrix, where it is oxidized and decarboxylated by pyruvate dehydrogenase, forming acetyl-CoA, which can enter the Krebs cycle. The monocarboxylic acids (pyruvate, lactate) can be transported out of Sertoli cells via the family of proton-linked plasma membrane transporters known as MCTs. Abbreviations: ALT, alanine aminotransferase; GLUT, glucose transporter; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter

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Chapter 2

Hormonal control of male reproductive function

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Hormonal control of male reproductive function

Introduction

Reproduction is a key process for the survival of a specie thus being subjected to a tight control. Hormones are the main players in this regulation which act as dynamic signaling molecules that modulate crucial events including gene transcription and translation, directly influencing the reproductive phenotype. The most known hormones are those belonging to the hypothalamic-pituitary gonadal (HPG) axis, which is based on the interaction between the hypothalamus, pituitary and the testes [1]. In brief, gonadotropin releasing hormone (GnRH) is synthesized by hypothalamus, which stimulates the pituitary to produce gonadotropins: the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH). LH binds to membrane receptors on Leydig cells and stimulates testosterone production. Whereas, FSH binds to membrane receptors on Sertoli cells, stimulating production/secretion of 17β-estradiol, activin and inhibin B [2]. The 17β-estradiol acts on Leydig cells to inhibit the production of testosterone [3]; and activin produces a positive feedback [4] while inhibin B and 17β-estradiol produce a negative feedback on pituitary [4-7].

On the last few years, several other intervenient in the control of male reproductive function have been unveiled. In fact, male fertility is also known to be dependent of overall metabolic and energetic homeostasis [8]. Although testicular metabolism was overlooked for decades, the adequate overall body metabolic functioning and particularly in testicular cells is crucial for the normal occurrence of spermatogenesis. Nowadays this emerged as a hot research field and many other hormones associated with all body metabolic regulation were found to influence male reproductive function [9]. In fact, insulin, which is linked with glucose homeostasis, is now known as an important regulator of spermatogenesis [10]. Similarly, gut hormones have been described as important regulators of male reproductive potential, besides its well-known role in controlling feeding/fasting status [11]. Moreover, as adipose tissue was recognized as an endocrine organ, the hormones that it produces, the adipokines, were also reported to interact with several other body systems, namely the reproductive system. Thyroid hormones (THs) also influence the reproductive function of males [12]. In fact, they are so important for the modulation of male reproductive potential, that any fluctuation in thyroid hormone levels, either hypo- or hyperthyroidism may lead to impaired spermatogenesis. During this chapter, we will discuss the most relevant hormones and pathways that interact with male reproductive system.

The hypothalamic-pituitary-gonadal axis

The HPG axis is formed by the hypothalamus, pituitary and gonads (testes in the male) (Figure 1). This axis is the key hormonal control system that modulates spermatogenesis and all the events occurring on the male reproductive tract. The hypothalamus synthesizes and releases neuro-hormones, namely GnRH [1]. The mammalian GnRH is a peptide hormone synthesized and released from GnRH-producing neurons within the arcuate nucleus of the hypothalamus [13]. Still, this secretion is not uniform during life, and changes happen during sexual development [14]. GnRH-producing neurons have an intrinsic pulse-generating ability leading to a pulsatile release of GnRH [15]. This pulsatile frequency and the concentration of GnRH influences the subsequent release of LH and FSH [16] having a crucial role in maintaining a normal steroidogenesis and gametogenesis. After its secretion, GnRH enters to the hypothalamic-pituitary portal system and binds to GnRH cell membrane receptors (GnRHR) on gonadotropic cells. These cells are located in the adenohypophysis, which represents 80% of the pituitary gland. The GnRHR is a transmembrane protein and its levels are regulated by GnRH. In fact, GnRHR levels increase when endogenous GnRH is increased. However, a continuous exposure to GnRH leads to desensitization causing a GnRHR downregulation [17]. When GnRHR is activated, it triggers several signal transduction pathways that lead to the release of the gonadotropins LH and FSH [18].

As GnRH, these gonadotropins are released in a pulsatile manner. However, while slower GnRH pulses lead to FSH synthesis, faster pulses favor LH synthesis and release [19]. An increase in the amplitude of LH pulses marks the beginning of puberty and the reactivation of reproductive axis, stimulating the secretion of gonadal sex steroid hormones. As puberty progresses, testosterone starts to control GnRH release, maintaining the frequency of LH pulses [20]. The major androgen produced in the testes is testosterone, a key regulator of spermatogenesis. This hormone is produced by Leydig cells in response to LH and targets Sertoli cells (Figure 1). These cells have androgens receptors (ARs) located in the nucleus and cytoplasm [21-23]. The other pituitary hormone secreted in response to GnRH is FSH, that binds to G-protein coupled receptors, exclusively located in Sertoli cells [2] (Figure 1). The activation of FSH receptor results in an increase of cyclic AMP signaling pathway, leading to increased levels of phosphorylated protein kinase B (PKB-P) through a phosphatidylinositol 3-kinase (PI3K)-dependent mechanism [24] and to the production and secretion of inhibin B by Sertoli cells.

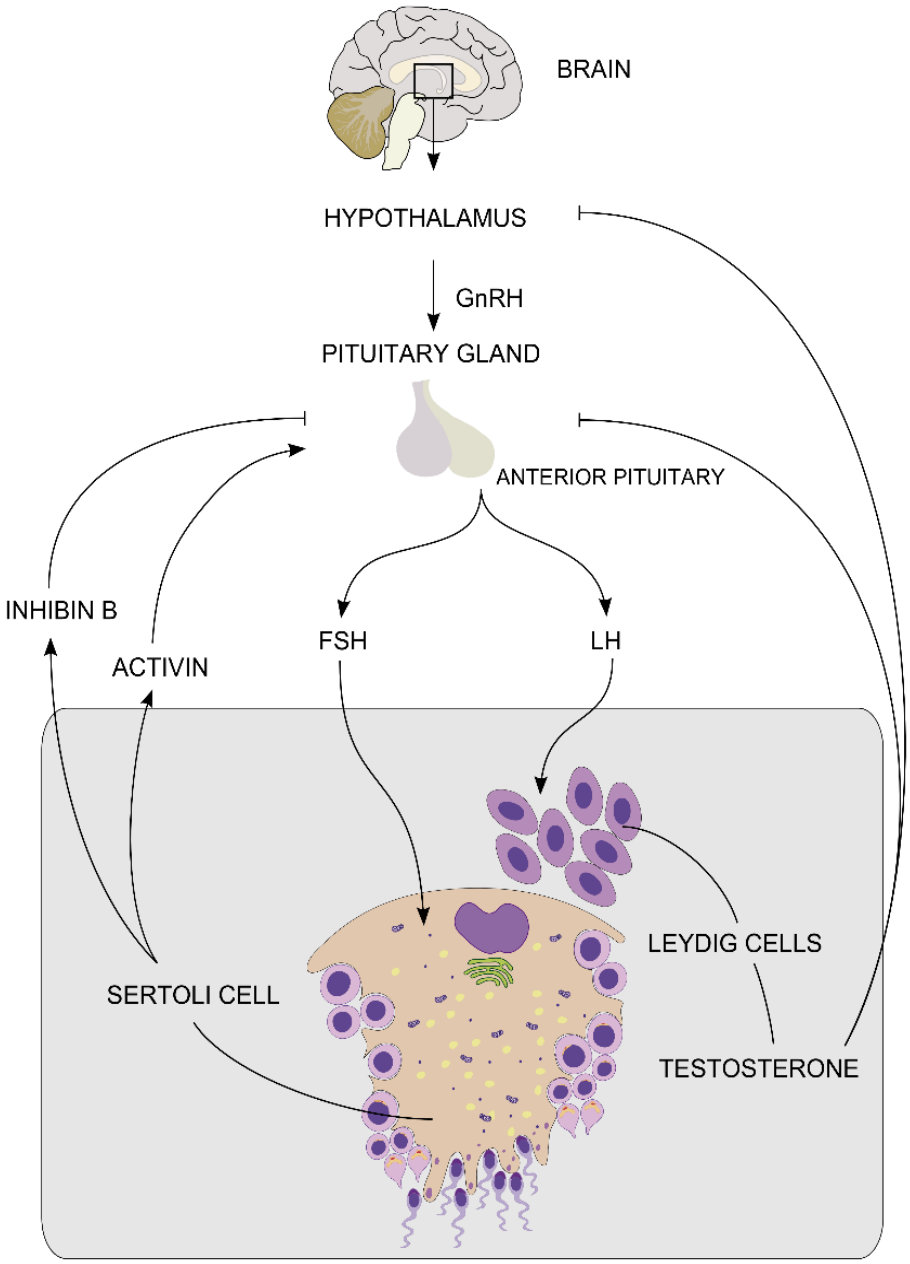

Figure 1. Schematic illustration of the hypothalamic-pituitary-gonadal axis. GnRH released by the hypothalamus stimulates the synthesis and release of the gonadotrophins FSH and LH by the anterior pituitary. These gonadotrophins are transported to the testis where FSH stimulates inhibin production by Sertoli cells. Inhibin has a negative feedback effect on the pituitary release of FSH. Sertoli cells may also secrete activin to increase FSH secretion. LH stimulates testosterone production by the Leydig cells. After entering circulation, testosterone is transported to the hypothalamus and exerts a negative feedback effect on hypothalamic GnRH release and has a direct negative effect on the release of FSH and LH by the pituitary. Abbreviations: FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone

Inhibin B is a member of the transforming growth factor β family of proteins and its co-receptor betaglycan binds to the activin type II receptor, blocking its association with activin. Therefore, it selectively inhibits FSH biosynthesis and secretion through a negative feedback controlled by GnRH [25] without affecting LH secretion [26] (Figure 1). Inhibin is a dimer composed by a α subunit and either a β_{A-} or a β_B-subunit. In contrast, activin, a dimer composed of two identical inhibin β subunits, plays a positive feedback to the anterior pituitary and stimulates FSH release [27, 28] (Figure 1). In brief, activin binds to serine-threonine kinase membrane receptors on pituitary gonadotroph cells. This leads to the release of FSH and to the transcriptional activation of *FSHB* and *GnRHR*, genes encoding for the β subunit of FSH and the receptor of GnRH. Therefore, the number of GnRHR on the cell membrane upsurges thus increasing the expression of the gene that codifies for LH (*LHB)* and LH secretion in response to GnRH.

Growth hormone

Growth hormone (GH) is a hormone produced by the somatotropic cells of the anterior pituitary in response to hypothalamic growth hormone releasing hormone [28]. GH exerts its functions, namely in steroidogenesis, gametogenesis, and gonadal differentiation, through its receptor, growth hormone receptor (GHR). In fact, GH is thought to play an important role in testicular differentiation and development for several reasons. First, a lack of GH secretion in adult rats results in a delay in testes growth and in the differentiation of the germ cells [29]. Moreover, administration of GH blockers stops the differentiation of the Wolffian duct in male fetal mice, whereas GH administration in female fetuses stabilizes the Wolffian duct [30]. GH also has a role after birth, namely in gametogenesis. Sperm motility, concentration and morphology are reduced in GH-deficient animals but restored upon GH administration [31, 32] illustrating the relevance of GH to those mechanisms although they remain to be unveiled. However, it is known that GH binds GHRs on Sertoli cells and increases the synthesis and/or modification of proteins such as insulin-like growth factor 1 (IGF-1), IGF-binding proteins, that mediate the stimulatory effects of GH on spermatogenesis. In what concerns to steroidogenesis, it is primarily regulated by LH. However, GH binds Leydig GHRs, activates second messengers, and stimulates the activity of several steroidogenic enzymes directly and by increasing LH receptor abundance [33].

Androgens

Androgens are regarded as the key responsible for the establishment of the sexual male phenotype [29], and belong to the group of steroid hormones. They are essential in the male sexual differentiation, in the development and maintenance of secondary male characteristics and

for the initiation and development of spermatogenesis [29-31]. Testosterone and 5α-dihydrotestosterone (DHT) are the two major androgens responsible for the referred functions. Although these androgens bind to the same receptor, they have different and specific roles in male sexual differentiation. Testosterone is responsible for the development of the embryonic Wolffian duct derived structures (epididymis, *vas deferens*, seminal vesicles and ejaculatory ducts), spermatogenesis, secondary sexual characteristics, such as muscle development, voice deepening, and axillary and pubic hair growth [32, 33]. On the other side, DHT, a metabolite of testosterone, plays a crucial role in prostate development and growth, development of external genitalia and male patterns of facial and body hair growth or male-pattern baldness [33, 34].

Cholesterol is the precursor for the synthesis of steroids hormones. The testes have the ability to convert this organic molecule to androgens. The steroidogenic acute regulatory protein (StAR) transports cholesterol to the inner membrane of mitochondria, making this a limiting step during steroidogenesis [35]. Inside the mitochondria, the side chain cleavage system comprised of CYP11A1, ferrodoxin and ferrodoxin-reductase are responsible for the conversion of cholesterol to pregnenolone. It is then translocated from the mitochondria and transported to the smooth endoplasmic reticulum, where it is converted into testosterone and finally secreted by the Leydig cell [36]. Once on circulation, this hormone exerts a negative feedback on hypothalamus (Figure 1) [37].

The action of testosterone and DHT is mediated by the AR, a member of the nuclear receptor superfamily [38, 39]. Although the complete structure of AR is not fully elucidated, it is known that AR is structurally divided into functional domains: a N-terminal domain (NTD), a DNA binding domain (DBD), a C-terminal ligand binding domain (LBD), and a minor hinge region between DBD and LBD. A huge part of AR transcriptional activity is mediated by NTD, which is the most active co-regulator interaction surface. The interaction of DHT and testosterone with AR is different. DHT has a two-fold higher affinity to the AR than testosterone. However, the dissociation of testosterone to the receptor is five-fold slower than DHT [33].

Estrogens

The connection between male function and estrogens has been under discussion for decades. Nowadays, it is widely accepted that estrogens have an important role in the development and maintenance of male fertility [40, 41]. In fact, estrogens are produced in the testis, being immature Sertoli cells the major source of estrogens during pre-puberty, while in adult life the major producer of estrogens is the Leydig cell [42, 43]. One of the main targets for estrogens is the Leydig cell, inhibiting the production of testosterone [44]. In the 30’s it was described that C19

androgens could be directly transformed in C18 phenolic estrogens. Later, the enzyme responsible for this reversible reaction was described and named aromatase or cytochrome P450 aromatase (P450arom) [45]. This enzyme belongs to the cytochrome P450 superfamily [46] and is composed by two polypeptides, a specific P450arom encoded by the *CYP19* gene and a ubiquitous NADPH cytochrome P450 reductase. In addition to be expressed in Sertoli and Leydig cell, the aromatase is also expressed by germ cells [47, 48]. The aromatization of androgens in order to synthesize estrogens englobes three reactions: sequential hydroxylation on the C19 methyl group and demethylation that lead to the removal of C19 as formic acid and the aromatization of the steroid A-ring, a characteristic of C18 estrogens [49]. Dehydroepiandrosterone, testosterone and androstenedione are the main aromatase substrates. In human male reproductive tract, Leydig [50, 51] and Sertoli cells [51, 52] are reported as estrogens producers. The 17β-estradiol produced by these cells has a negative feedback on pituitary [6].

The biological effects of testicular estrogens are mediated by estrogen receptors (ERs). Estrogens diffuse in the cell and bind to nuclear ERs, forming an estrogen-ER complex, modulating transcriptional target genes. In testicular cells, estrogens are important on male gamete maturation and three ERs known to mediate estrogen effects are present in these cells: ERα [53], ERβ [54] and G-protein-coupled estrogen receptor 1 (GPER) [55]. ERα and ERβ are nuclear receptors (NRs) of a family of ligand-modulated transcription factors [56-58]. The expression of ERα and ERβ is now globally accepted in the testicular tissue. The full-length ERα (66 kDa) and one isoform lacking the exon 1 (46 kDa) were found in immature male germ cells, and the 46 kDa isoform was observed in spermatozoa. ERα can also be found in Leydig cells [59, 60] and in the cells of the epithelium of efferent *ductus*, the region linking *rete* testis to the head of the epididymis [60-62]. The expression of this receptor was also found in spermatocytes, spermatids and spermatozoa [40, 48, 63, 64]. As concerning ERβ, two proteins, which correspond to a long (60 kDa) and short (50 kDa) forms of ERβ were identified in germ cells [65]. The expression of ERβ has also been described in Leydig [66], Sertoli [67] and peritubular cells [66, 67]

GPER, also known as G-protein coupled receptor 30, is an orphan G-protein coupled receptor with seven transmembrane domains [68]. A study performed in 2000 found that rapid 17β-estradiol-mediated activation of extracellular signal-regulated kinases (ERKs) was dependent on GPER, being the most recent known ER [55]. It is located on cell surface and is both, structurally and genetically distinct of ERα and ERβ. It is responsible for a quick activation of different pathways, leading to the rapid phosphorylation of the mitogen-activated protein

kinases ERK1/2 [55, 69], stimulation of adenylyl cyclase [70, 71], PI3K signaling pathways activation and mobilization of intracellular calcium stores [72]. The expression of GPER was demonstrated in male reproductive tract, namely in Sertoli cells, diploid germ cells, epididymis, *vas deferens*, seminal vesicles and prostate (for review [73]). However, GPER is not present in haploid germ cells [74].

Insulin

Insulin is a peptide hormone secreted by pancreatic β cells located in the islets of Langerhans [75]. Since its discovery, insulin has been considered a key circulating signal for energy homeostasis. In fact, this peptide has a crucial role in the physiological maintenance of blood glucose levels, and its dysregulation may lead to the onset of several metabolic disorders. Due to its importance, the exocytosis of insulin from β cells is a tightly regulated process.

The effects of insulin on peripheral tissues, and specially on male reproductive system, are mediated by the insulin receptor, which is widely expressed in testicular tissue, more specifically in Sertoli, Leydig and peritubular cells [76, 77]. When insulin binds to its receptor, the latter is phosphorylated and some effector proteins, namely PI3K are recruited and activate several signaling pathways [78]. These are involved in different cellular processes, such as metabolism differentiation and proliferation [79]. Although the specific molecular mechanisms remain to be unveiled, it is known that insulin affects male reproductive function at multiple levels. In fact, besides energy homeostasis, insulin is necessary for testicular embryonic development [80]. When the family of insulin receptor tyrosine kinase are absent, XY mice develop ovaries and a female phenotype, indicating that insulin signaling pathway is required for male embryonic development [80]. In addition, ablation of insulin receptor leads to decreases in both testes size and sperm production due to reduced proliferation rate of immature Sertoli cells during late embryonic period [81]. HPG axis functioning is also modulated by insulin. GnRH secretion is sensitive to metabolic signals and pathological situations that lead to hypoinsulinemia or hyperinsulinemia which are commonly linked with disturbed GnRH/LH pulses [82]. Indeed, exogenous insulin administration was found to increase LH that in turn promotes testosterone synthesis in Leydig cells [83]. In sum, besides energy homeostasis, insulin modulates the male reproductive system in several ways, even before birth. However, some of the mechanisms that lead to these effects remain to be unveiled.

Thyroid Hormones

Thyroid hormones (THs) are crucial for the homeostasis maintenance of some processes, particularly the control of body overall metabolism, protein synthesis, fat metabolism, neural development, normal growth and maturation of bones, as well as renal and cardiovascular functions [84, 85]. The thyroid gland synthetizes THs through iodination of tyrosine residues of the glycoprotein thyroglobulin [86, 87]. The hypothalamus and pituitary gland are the major controllers of THs production. Neurons in the paraventricular nucleus of the hypothalamus secrete thyrotropin-releasing hormone that stimulates the pituitary gland to secrete thyrotropin/thyroid-stimulating hormone (TSH). In turn, TSH binds to thyroid follicular cell basolateral membrane, which express a G-protein-coupled TSH receptor [88]. The prohormone thyroxine (3,3',5,5'-tetraiodothyronine or T4) is the main product of the thyroid gland, but it has low biological activity. Through the intracellular removal of an iodine atom from the outer-ring of T4, 3,30,5-triiodothyronine (T3), the biologically active hormone is originated. The iodine uptake is regulated by TSH and is mediated by the sodium/iodide symporter. T3 has 100-fold higher affinity to thyroid hormone receptor (THR) than T4 [89, 90], so the reaction catalyzed by type 1 and type 2 iodothyronine deiodinases is essential. After its biosynthesis, THs enter the blood stream and bind to plasma thyroid hormone binding proteins in order to be distributed, since they are hydrophobic. However, THs need transporters to cross cell membranes and reach target cells, being the monocarboxylate transporter 8 the most important transporter to perform that duty [91]. The effects of THs are mediated by a nuclear THR that belongs to the superfamily of NRs, and modulates gene transcription in response to hormone binding. Recognizing specific nucleotide sequences, the nuclear response elements, THRs bind to the promoter region of target genes and recruit co-activators or co-repressor proteins [56, 92, 93]. In human Sertoli cells, THRα1 and THRα2 have already been described [94]. In rat Sertoli cells, T3 suppresses the expression of immature Sertoli cell markers [95, 96]. On the other hand, hypothyroidism extends the expression of these markers in neonatal rats [95], showing a role of T3 in the maturation of Sertoli cells. Moreover, T3 regulates gap junctions between Sertoli cells through an increase of connexin 43 levels, a gap junction protein [97]. THs are also very important for Leydig cells. Indeed, after elimination of Leydig cells population in rats, T3 treated rats have a faster and superior recovery of Leydig cells population when compared to controls with normal concentrations of THs [98]. Moreover, THs also influence steroidogenesis, increasing the production of testosterone [99, 100]. During germ cell development, THRα is expressed in type B spermatogonia and THRβ1 is expressed in intermediate type spermatogonia [12], suggesting a role for THs in sustaining

different populations of germ cells. Along the male reproductive tract, THRα1 and THRβ1 are also expressed in the epididymis [101].

Gut Hormones

Ghrelin

Ghrelin, as well as obestatin, is a gastrointestinal hormone obtained by post-translational processing of its precursor, preproghrelin [102]. Preproghrelin is composed by a 23 amino acids signal peptide and by proghrelin, a 94 amino acids peptide [103]. Ghrelin is a 28 amino acids segment of proghrelin, which is also composed by a carboxy-terminal peptide, the C-ghrelin [104-106]. Besides ghrelin, the proghrelin can be also processed to obestatin [107]. Ghrelin is subjected to posttranslational modifications comprising the acylation of the hydroxyl group of the Ser3 [108]. This acylation is required to activate the ghrelin receptor, also known as growth hormone secretagogue receptor (GHS-R), and to mediate its effects on growth hormone secretion and food intake [109]. However, acylated ghrelin represents less than 10% of the circulating ghrelin [105, 110, 111]. Nevertheless, it has been hypothesized that desacylated ghrelin has a role in modulating the effects of acylated ghrelin.

The effects of ghrelin in the reproductive axis have been discussed for several years, and some studies suggest that this hormone is an important modulator of male reproductive function at different levels. Firstly, this hormone acts on the HPG axis, inhibiting the secretion of GnRH, LH and FSH [112-115] and stimulating the secretion of prolactin [116]. Moreover, in male rats it has the capacity to delay puberty onset [117] and downregulate *Kiss1* [118], which encodes for a protein that stimulates the secretion of GnRH. The presence of GHS-R has been demonstrated in human germ cells, pachytene spermatocytes [119], Leydig [119] and Sertoli cells [11, 119]. Moreover, ghrelin has been detected in Sertoli and Leydig cells [119]. Additionally, in cases of varicocele, obstructive azoospermia or normozoospermia the serum levels of testosterone are inversely correlated with ghrelin levels, suggesting an effect on spermatogenesis [120]. In normal fed rats, ghrelin decreases testicular mass, but does not alter testosterone levels, while in rats subjected to food restriction, ghrelin decreases testosterone plasma levels [121]. An *in vitro* study, using adult rat testicular tissue, demonstrated that ghrelin inhibits human chorionic gonadotropin- and cAMP-stimulated testosterone secretion [122]. This inhibitory effect of ghrelin is associated with a decrease in human chorionic gonadotropin-stimulated expression of mRNAs encoding StAR, and P450 cholesterol side-chain cleavage, 3β-hydroxy steroid dehydrogenase, and 17β-hydroxy steroid dehydrogenase type III enzymes [122]. There is also evidence that ghrelin affects the development and maintenance of testicular cells. In fact, ghrelin has an inhibitory action in

stem cell factor mRNA expression, a crucial signal for the development of Leydig cell and germ cells production [119, 123] (Figure 2). Therefore, the effect of ghrelin on stem cell factor expression may disturb spermatogenesis and Leydig cell proliferation. More recently, the effects of ghrelin in cultured Sertoli cell suggested that this hormone can act as an energy status sensor for the male reproductive system. Moreover, there is an inverse association of this hormone with the production of lactate, thus controlling the nutritional support of spermatogenesis [11]. Hence, ghrelin acts as a regulator of testicular development and function, since this hormone modulates Leydig cells development and function as well as Sertoli cell metabolism.

Glucagon-like peptide-1

Secreted mainly from entero endocrine L-cells in the epithelium of the distal ileum and colon, but also from cells in the pancreas, and in the central nervous system [124], glucagon-like peptide 1 (GLP-1) belongs to gastrointestinal peptide hormone incretin family [125-127]. GLP-1 has several effects on pancreatic β cells, such as stimulation of glucose-induced secretion of insulin and β cell neogenesis, besides inhibiting β cell apoptosis. Moreover, GLP-1 delays gastric emptying and increases peripheral glucose disposal [128, 129]. Furthermore, as GLP-1 can cross the blood-brain barrier [130], it acts in the brain in order to enhance satiety. GLP-1 binds to its receptor, GLP-1R, a member of a glucagon receptor family of G protein-coupled receptors [131], which is widely expressed in the pancreatic islets, heart, brain, kidney and stomach [132-137]. The human GLP-1 receptor (GLP-1R) has an N-terminal extracellular domain and some glycosylation sites, essential for the coupling of GLP-1 and for the successful trafficking and processing of the receptor [138-140].

GLP-1 was identified in 1981 in the translational products of mRNAs isolated from the pancreatic islets of anglerfish being the second incretin to be discovered [141, 142]. GLP-1 is part of the product of proglucagon gene, and has approximately 50% of homology to glucagon [143]. In circulation, there are two types of bioactive GLP-1 being GLP-$1_{7\text{-}36\ \text{amide}}$, which corresponds to proglucagon 78–107 with its C-terminal Arg amidated, the most common circulating form in humans [144, 145]. However, the half-life of this hormone is very short, since it is cleared by the kidney and inactivated by the ubiquitous proteolytic enzyme dipeptidyl peptidase-4, which cleaves two amino acids from the n-terminus of GLP-1 [146].

Although it is known that GLP-1 has a key role in maintaining glucose and nutrient homeostasis, little is known about the effects of GLP-1 in male reproductive function. However, GLP-1 has several effects in the central nervous system that may impair HPG axis. Moreover, the administration of GLP-1 in healthy men results in a decrease of testosterone production

independent of LH levels, illustrating a role of GLP-1 in steroidogenesis [147]. In addition, GLP-1 seems to have a role in puberty onset, since it alters the secretion of hypothalamic GnRH [148] (Figure 2). The same study showed, through a knockout mouse model of GLP-1 receptor, that even though the levels of gonadal sex steroids were normal, the males have decreased gonadal weight [148]. Although more studies are needed to unveil the mechanisms by which GLP-1 affects male reproductive function, it is already clear that this hormone may modulate by several mechanisms.

Adipokines

Leptin

Leptin is a 16 kDa adipocyte-derived protein encoded by the obese (*ob*) gene [149]. Besides adipocytes, leptin is locally produced in other tissues, such as ovary, skeletal muscle and stomach [150, 151]. This may suggest pleiotropic effects of leptin on several biological functions. In fact, leptin plays a key role in appetite modulation, but was also reported it has a role in immune and gonadal functions and stress responses [152-154]. As leptin is mostly produced in white adipose tissue, its concentration in the blood stream is positively correlated with the amount of fat mass of an individual.

After being secreted by adipocytes, leptin circulates in the bloodstream bound to a circulating isoform of LepR. Then, it surpasses the blood-brain barrier *via* the short leptin receptor isoform to the hypothalamus [155]. Here, leptin binds to its receptor (LepR) on neurons producing anorexigenic proopiomelanocortin (POMC) and Agouti-related protein (AgRP) within the arcuate nucleus. Leptin binds to LepR and signal transducer and activator of transcription 3 is activated, which will then bind to *pomc* and *agrp* promoters, activating the first and inactivating the second one [156]. Besides, leptin also activates PI3K, which in turn induces the synthesis of PIP_3. The accumulation of the latter leads to 3-phosphoinositide-dependent protein kinase 1 and protein kinase B activation resulting in the inactivation forkhead box protein O1 that functions as inhibitor of POMC expression and activator of AgRP [157]. This will then suppress energy intake and stimulate energy expenditure. Consequently, leptin is known as the satiety hormone.

As leptin has a key role in the regulation of nutrient homeostasis, this hormone also impacts male reproductive function, mainly through its neuroendocrine effects. In fact, leptin deficiency leads to pubertal failure and infertility [158]. On the other hand, in obese men, the increased concentration of leptin inhibits testosterone production by Leydig cells, impairing spermatogenesis [159]. This effect may be due to the capacity of leptin to decrease testicular expression levels of StAR and cholesterol side-chain cleavage enzyme, upstream elements of the steroidogenic pathway, in a

dose-dependent manner [160]. Moreover, leptin was found to regulate estrogen synthesis through modulation of aromatase namely in the prostate [161]. In addition, leptin also causes alterations in testicular physiology. Hyperleptinemia causes a decrease in the number of spermatocytes and sperm resulting in a decrease in the diameter of the seminiferous tubules. Likewise, the number of Leydig cells is also decreased as well as testis weight [162] (Figure 2). In what concerns to Sertoli cells, it was recently found that leptin modulates the nutritional support of spermatogenesis [154]. Furthermore, leptin crosses blood-testis barrier [163] and its levels in the seminal plasma are inversely correlated with normal sperm parameters [164]. Nonetheless, it is reported that leptin has no effects on motility, capacitation and acrosome reaction of human spermatozoa [165].

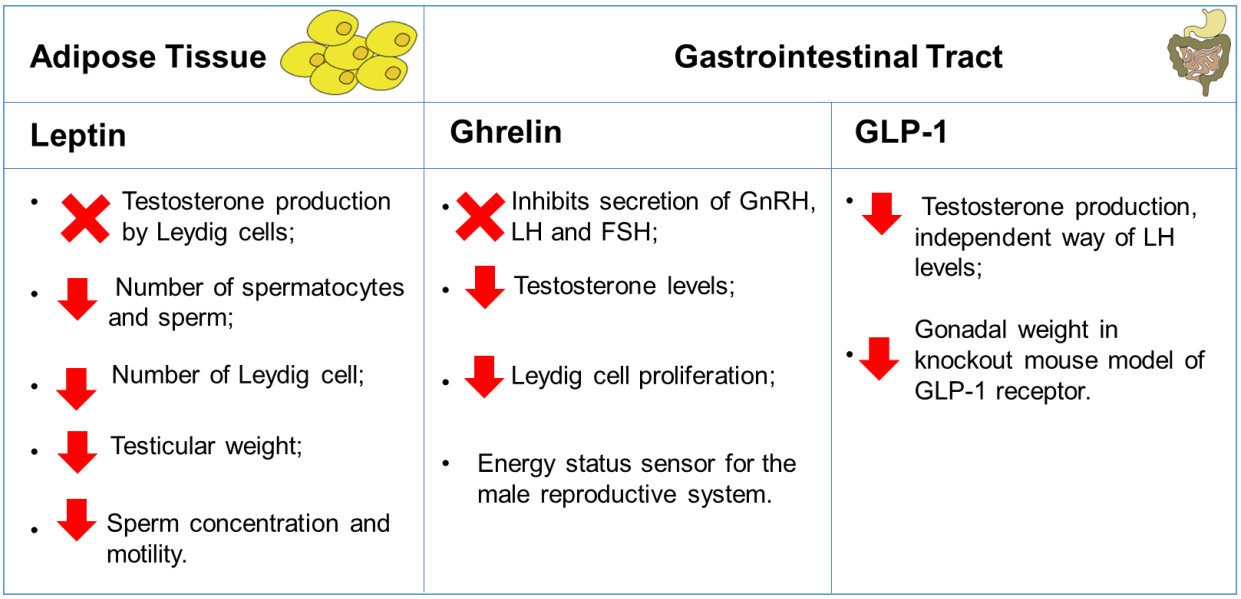

Figure 2. Effects of leptin, ghrelin and glucagon like peptide-1 (GLP-1) on male reproductive tract and reproductive health. Leptin is produced by the adipose tissue and decreases testosterone production resulting in decrease of sperm concentration and motility. Ghrelin is produced in the gastrointestinal tract and affects the hypothalamic - pituitary - thyroid axis, affecting Sertoli and Leydig cells. GLP-1 also produced in gastrointestinal tract decrease testosterone levels and testicular weight. FSH, follicle stimulating hormone; LH, luteinizing hormone; GnRH, gonadotropin-releasing hormone.

Concluding Remarks

Reproductive function, as well as the development of secondary sexual characteristics, is highly regulated. In fact, normal testicular function is dependent upon the action of hormones through both endocrine and paracrine pathways. As discussed, HPG axis is the major intervenient known to affect and control male reproduction. The key roles played by hormones involved in this axis in the regulation of steroidogenesis and spermatogenesis are known for several decades.

Androgens, which exert feedback actions on the hypothalamus and anterior pituitary, also have a crucial role for spermatogenesis. These hormones may also be converted into estrogens, which have been acquiring attention since they were also found to have a key role in male reproduction. However, some new intervenient have been discovered in the last few years. The drastic increase of metabolic disorders, and its association with the decrease in fertility rates, has brought attention to this topic and thus, the hormones related with those processes are also on spotlight. In fact, these may be one of the missing links between the disease and the already known consequences of impaired reproductive function in males.

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Chapter 3

Objectives

Objectives

Infertility is a worldwide problem and is considered by the World Health Organization (WHO) a disease where in one-third of the cases the contributor factor is attributed to the male partner. Spermatogenesis is a complex process whereas spermatogonia differentiates into spermatozoa. Inside the testis, Sertoli cells are the key somatic element responsible for spermatogenesis, as they ensure, among other functions, the nutritional support of the developing germ cells.

Sertoli cells are tagged as major hormonal targets, and recent studies by our group demonstrated a modulation of these cells by hormones associated with whole body metabolism, particularly insulin. Furthermore, other recent studies relate the increased incidence in metabolic diseases with the observed decrease in fertility rates we are witnessing in modern societies.

Obesity is a worldwide health problem and its prevalence is increasing particularly due to a change in life habits, such as, consumption of high fat diets or sedentary lifestyle. The development of overweight and obesity is caused and can be a consequence of hormonal dysregulation, which can also influence the nutritional support of spermatogenesis by Sertoli cells. In this work, we aimed to study the effects of hormonal dysregulation associated with food intake in Sertoli cell metabolism and bioenergetics. This study also aimed to elucidate the effects of a caloric restriction by using an animal model to study sperm parameters and testicular metabolism. There were three major goals for this project:

1. Leptin and ghrelin are responsible for the regulation of appetite, energy metabolism and fat distribution in the body. The effects of leptin and ghrelin dysregulation in fertility were previous discussed. However, the molecular mechanisms by which it regulates male reproductive function remain to be elucidated. Thus, we aimed to explore the effects of leptin and ghrelin in Sertoli cell metabolism and consequently in the nutritional support of spermatogenesis.
2. Glucagon-like peptide-1 (GLP-1) based therapies are well-established pharmacological resources for obesity treatment. GLP-1 functions include energy regulation and glucose homeostasis, which are essential for spermatogenesis. In this study we aimed to explore GLP-1 effects in human Sertoli cells metabolism and mitochondrial function.
3. In conditions of low energy available, for example in case of caloric restriction (CR), health problems can arise. The effects in male fertility remain controversial and the molecular

mechanisms by which CR affects male fertility remain to be elucidated. Thus, we aimed to explore the effects of a CR in testicular metabolome and sperm quality.

Hence, this thesis aimed to establish a link between the male reproductive health and the hormonal (dys)regulation caused by overweight/obesity and disturbed eating habits, particularly in the levels of ghrelin, leptin, GLP-1 and after caloric restriction. We aimed to study if food intake-related hormones can regulate Sertoli cell metabolism and consequently, the nutritional support of spermatogenesis. Finally, these findings will be essential to elucidate the link between infertility and metabolic diseases associated by studying (dys)regulation in leptin, ghrelin and GLP-1 as well as severe alterations in food intake, such as in the case of caloric restriction.

Chapter 4

Assessment of Sertoli cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and sulforhodamine B assays

This chapter was adapted from the published work:

Martins AD, Oliveira PF, Alves MG (2019) Assessment of Sertoli cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and sulforhodamine B assays. Current Protocols in Toxicology. 81(9):e81 *(*DOI: 10.1002/cptx.85)

Martins AD was responsible for the bibliographic review in the literature, construction of the outline and writing of the manuscript.

Assessment of Sertoli cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and sulforhodamine B assays

Abstract

The correct functioning of Sertoli cells is pivotal for a successful spermatogenesis. They are major targets for hormones, endocrine disruptors and other substances that men are subjected to every day. One of the main Sertoli cells functions that quickly responds to a deleterious stimulus is proliferation. This is directly related with the *in vivo* capacity of these cells to sustain a good number of developing germ cells. The discussed protocols can be tested in Sertoli cells from different origin, in case of human Sertoli cells from small human testicular biopsies a short and simple protocol to isolate and culture these cells is included. The protocols discussed herein are two different procedures, somewhat complementary, to assess Sertoli cells proliferation. In brief, the sulforhodamine B assay allows to dye the healthy fixed Sertoli cells maintained in culture, while 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced by live Sertoli cells. These methods are mostly used to evaluate how Sertoli cells proliferative activity responds to the exposure to compounds such as toxicants or hormones.

Introduction

Sertoli cells are responsible for the formation of the seminiferous tubules and provide structural and nutritional support to the germ cells during spermatogenesis. Indeed, germ cells are dependent of the nutritional support provided by Sertoli cells, particularly due to the production of lactate by Sertoli cells to be used as energetic substrate by germ cells. Thus, it is imperative that Sertoli cells can function correctly to maintain an adequate pool of metabolites for germ cells [1]. The number of developing germ cells that each Sertoli cell can support is limited and thus, the reproductive toxicity of chemicals, as measured by Sertoli cell proliferation, has a direct impact in spermatogenesis. Therefore, several authors propose the study of Sertoli cells proliferation/cytotoxicity as a first assessment of possible alterations induced by compounds in spermatogenesis. To use human Sertoli cells from testicular small biopsies, a support protocol of isolation of these cells to further *in vitro* culture is included. To isolate Sertoli cells of rat, mice and bovine the protocol was previously well described [2]. When the option is to use commercial lines of Sertoli cells available it is recommended to follow the manufacture instructions.

There are some options available to evaluate the cytotoxicity of compounds, and most of those protocols work well on Sertoli cells. The use of reliable commercial kits is the simplest way to assess the cytotoxicity not only in these cells but also for most cell types. However, commercial kits are expensive, sometimes fall out of the detection range or are not sensitive enough. Herein we present two distinct, somewhat complementary protocols to assess cell proliferation in Sertoli cells, which are easy and cheap.

Sulforhodamine B (SRB) is an aminoxanthene dye that stoichiometrically binds to basic amino acid residues in proteins through the sulfonic groups. Thus, as the binding is stoichiometric, the changes in coloration directly represent the total protein mass and are proportional to the cell number. One major advantage of this protocol is that SRB staining is independent of the mitochondrial function, so there is not much optimization needed to adapt this protocol to different types of cells [3]. Regarding linearity and sensitivity, SRB is comparable to fluorescent dyes (DAPI or Hoeschst) and thus presents reliable outcomes [4]. In addition, compared with other colorimetric assays (Lowry, Bradford or azure A), sulforhodamine B presents even better linearity and higher sensitivity [5].

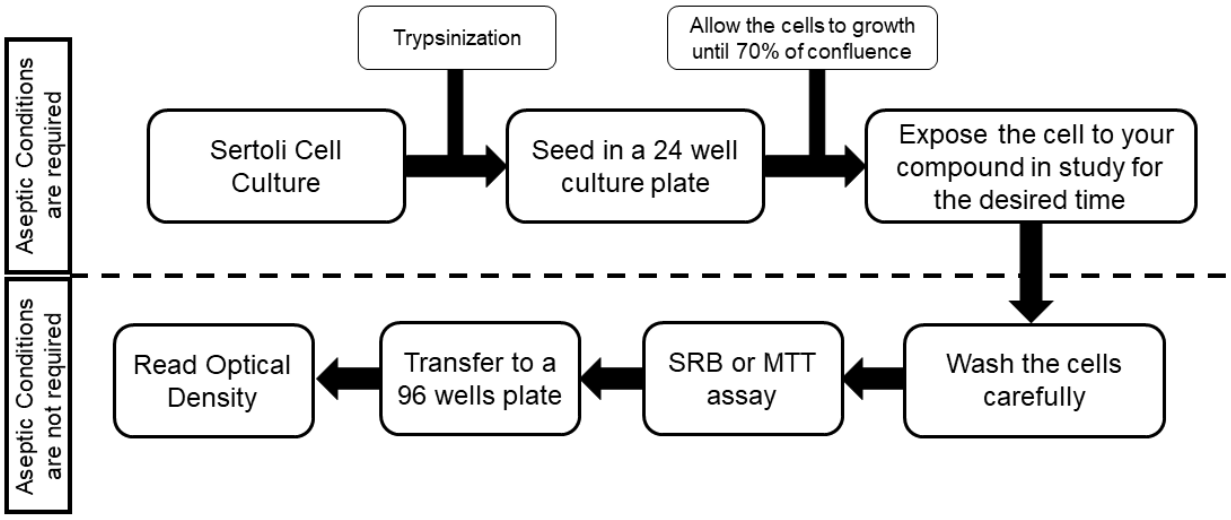

Figure 1. Schematic representation of a suggested workflow for the steps needed to perform 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or sulforhodamine B (SRB) protocols.

The second method in discussion is based on the use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a compound reduced by the viable cells into formazan precipitate, which must suffer a solubilization process to allow the read of the coloration optic density. In this protocol, the MTT precipitate that is formed is directly proportional to the number of viable cells. The basic principle of this assay is based on the fact that viable cells contain

NAD(P)H-dependent oxidoreductase enzymes, which are responsible for the reduction of MTT to formazan [6]. The required time for that reaction to occur can take at least 1 hour and go up to 4 hours, which is one of the major disadvantages of this assay. Other disadvantages should also be highlighted, including the interference of other reducing agents in the required reactions and the fact that MTT is toxic for the cells [7]. Figure 1 provides a flowchart of the steps that must be followed to perform the MTT or SRB assay.

Sulforhodamine B (SRB) assay

Sulforhodamine B (SRB) was developed in 1990 to be used at a large scale in *in vitro* studies focused on the anti-tumor effects of drugs [5]. SRB is an anionic aminoxanthene protein dye used for total protein and performed using *in vitro* measurements. In this assay, the dye binds to a basic amino acid of the cell proteins and through a colorimetric evaluation allowing to estimate total protein, which is directly related to the number of cells present in the preparation [4, 8]. Compared with other assays it has similar or higher linearity and sensitivity; it also does not require time-sensitivity measurements; and as a final advantage, it has reduced cost [9, 10]. The need for the cells to be fixed in the plate is the major disadvantage of the SRB assay, since the addition of methanol has to be carefully performed to prevent cells from detaching from the cell plate [10].

Materials List (Figure 2, Panel A)

- 24-well cell culture plate
- Phosphate-Buffered Saline Solution (see recipe in Reagents and Solutions)
- Fixing Solution (see recipe in Reagents and Solutions)
- 0.05% SRB Solution (see recipe in Reagents and Solutions)
- 1% Acetic Acid Solution (see recipe in Reagents and Solutions)
- Incubator at 37ºC
- 10 mM Tris solution (pH=10) (see recipe in Reagents and Solutions)
- 96-well plate
- Platform Shaker
- Multi-plate reader

Steps and Annotations

1. Seed the same number of Sertoli cells in each of the 24 well culture plate and allow them to grow until about 70% of confluence.
2. Expose the cultured cells to the compounds in study for the concentrations and time desired. Usually the compounds are directly dissolved in the cell culture (in distinct concentrations) while the vehicle is introduced in the control.
3. At the time point determined for the experiment, remove and discard the culture medium (Figure 2, Panel B), and carefully wash the cells one time with approximately 1 mL of phosphate-buffered saline solution at room temperature (Figure 3, Panel A and B).

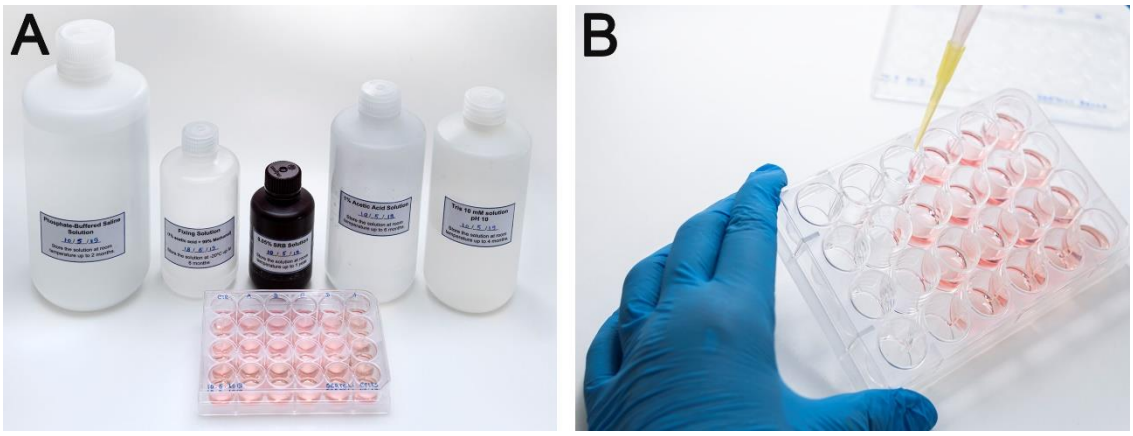

Figure 2. Setup with the solution necessary to the Sulforhodamine B assay (A). After the exposure of the Sertoli cells to the compound of interest, carefully remove the medium containing the compound (B).

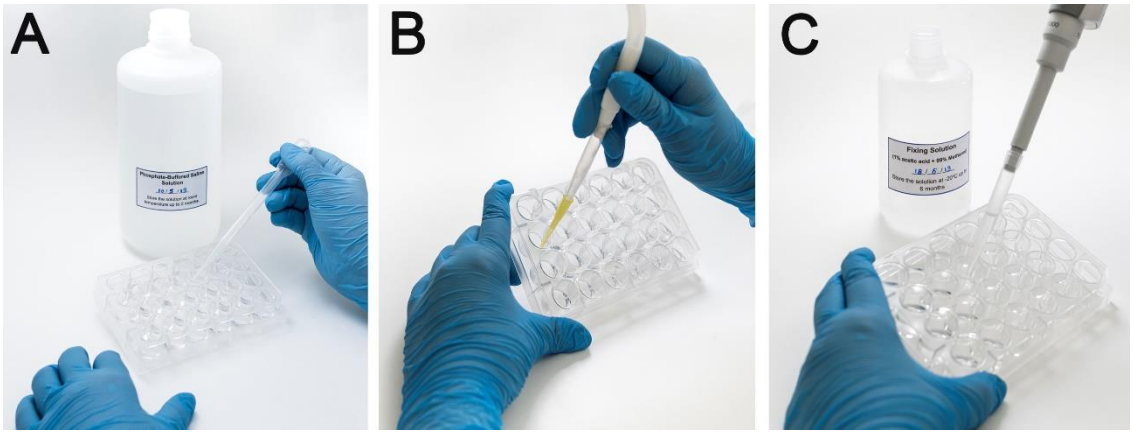

Figure 3. Wash the Sertoli cells with Phosphate buffered saline solution at room temperature (A), carefully remove the Phosphate buffered saline solution (B) and add the fixing solution (C) after at least 1 hour to -20ºC remove this solution.

4. Add 1 mL of fixing solution at -20ºC to each well (Figure 3, Panel C). Save the plate at -20ºC at least one hour.
5. Discard the fixing solution and allow the plate to completely dry at 37ºC in an incubator or at room temperature.
6. Add 500 μL of 0.05% SRB solution (Figure 4, Panel A) at each well and incubate 1 hour at 37ºC (Figure 4, Panel B).

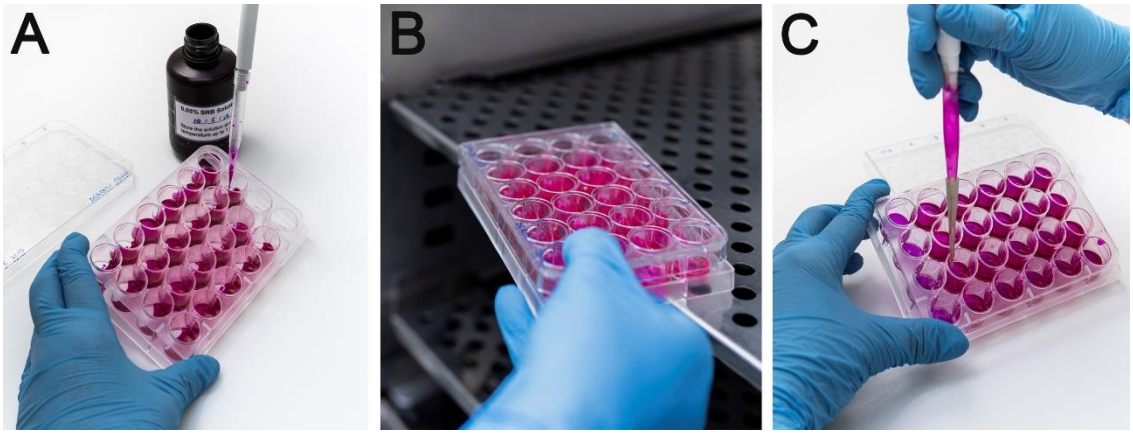

Figure 4. Allow the plate to dry and carefully add 0.05% Sulforhodamine B solution to cell culture plate (A), incubate for 1 hour at 37ºC in an incubator (B) and cautiously remove the SRB solution (C).

7. Discard the SRB solution (Figure 4, Panel C) and wash the wells with 1% acetic acid solution (Figure 5, Panel A) until no pink coloration is observed in the plate (this should be done at least 3-4 times).
8. Allow the plate to completely dry at 37ºC in an incubator or at room temperature, without the cover of the plate.

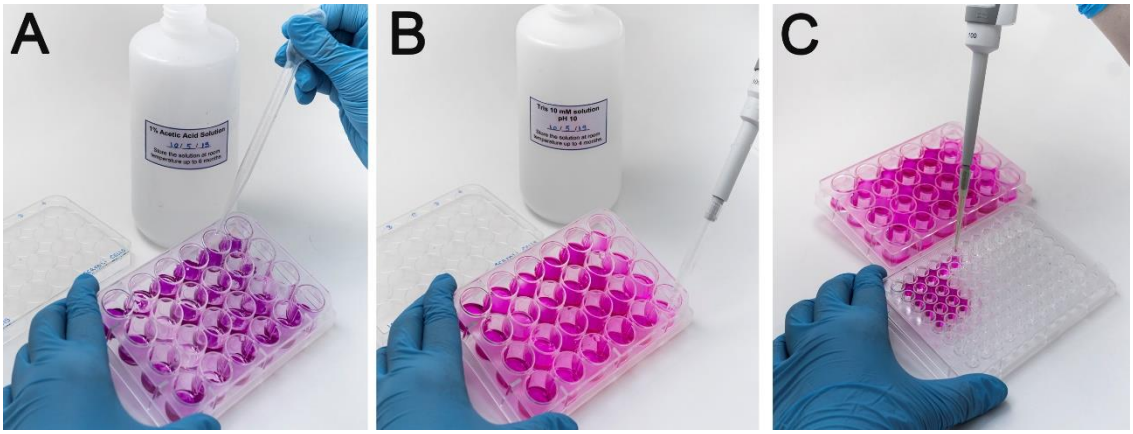

Figure 5. Add 1% acid acetic solution to each well of the cell culture plate (A), repeat this step until no pink coloration is observed when removing the acid acetic solution. Allow the plate to dry and add 10 mM Tris pH=10 to each well (B) and shake at room temperature. After 10 minutes transfer to a 96 multi-well plate (C).

9. Add to each well 500 μL of 10 mM Tris solution (Figure 5, Panel B) and shake the plate during 10 min to room temperature.

10. Remove 100 μL from each well to a 96 well plate (Figure 5, Panel C) (this should be done in triplicate to validate the readings). Use 100 μL 10 mM Tris solution to the blank (Figure 6, Panel A).

11. In a multi-well plate reader, read the absorbance at 565 nm (Figure 6, Panel B) (or in a range of 550 to 580 nm). If the objective is to attain a more intense colour, then set a suboptimal wavelength of 490-530 nm.

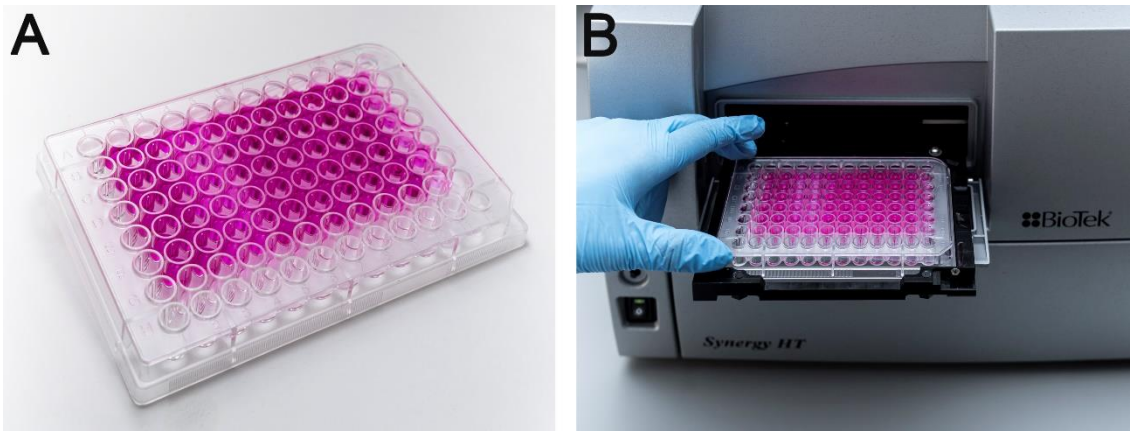

Figure 6. The coloration of the plate should be lighter, as the compound is more toxic to the cells (A), solution of 10 mM of Tris is used as blank. Read the optic density in a multi-well plate reader (B)

12. Data treatment: To assess Sertoli cells proliferation after an experimental treatment use the following data treatment:

I. If you performed the read in triplicates, calculate the average of the value for each condition

II. Remove the blank from all the experimental conditions:

$$Abs_{sample} = Abs_{read\ in\ the\ sample} - Abs_{blank}$$

III. Assuming the condition without any compound as your control (100%), calculate proliferation in the other conditions based on the control group by using the following data treatment:

$$\%\ Cell\ proliferation_{Condition\ A} = \frac{Abs_{Samples\ Condition\ A}}{Abs_{\ Samples\ Control}} \times 100$$

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was developed in the 1980´s to assess cell proliferation/toxicity of living cells through a 96-multiplate spectrophotometer reader [11]. This technique is based on the reduction of the MTT to formazan, in the living cells, by the dehydrogenase enzyme present in the mitochondria of the cells [12]. In fact, the reduction of MTT to formazan is directly proportional to the number of viable cells [11] and thus can be a valuable method to determine how the compound affects the cells proliferation *in vitro*.

MTT is water-soluble, resulting in a yellow solution. However once reduced, formazan is insoluble in water solution. In order to determinate the optic density, formazan crystals should be solubilized in one of the following: ethanol, propanol [13], mineral oil or DMSO [14]. One of the major drawbacks of this technique is the MTT incubation time to allow the reduction, which need at least 1h and can go up to 4h [7]. Similarly, to SRB assay, another disadvantage is the fact that the addition of the solution to the plate with the cells should also be carefully performed to avoid the detachment of the cells from the plate surface.

Materials List (Figure 7, Panel A)

- Phosphate-Buffered Saline Solution (see recipe in Reagents and Solutions)
- MTT Solution (see recipe in Reagents and Solutions)
- DMSO
- Incubator at 37ºC
- 96-wells plate
- Platform Shaker
- Multi-plate reader

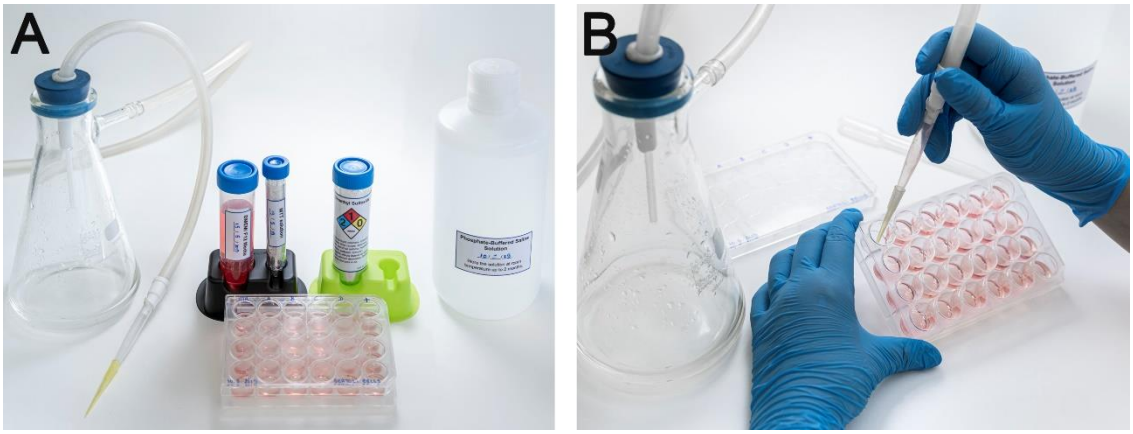

Figure 7. Suggested setup with the necessary solutions to the MTT assay (A). At the end of chosen time of exposure to the compound of interest, remove carefully the culture medium (B).

Steps and Annotations

1. Seed Sertoli cells in a 24-wells plate making sure you place the same number of cells in each well and allow them to grow until 70% of confluence.
2. Expose the cells to the compounds in test for the concentrations and time desired.
3. Remove and discard the medium (Figure 7, Panel B), then wash the cells very carefully with PBS at room temperature (Figure 8, Panel A).

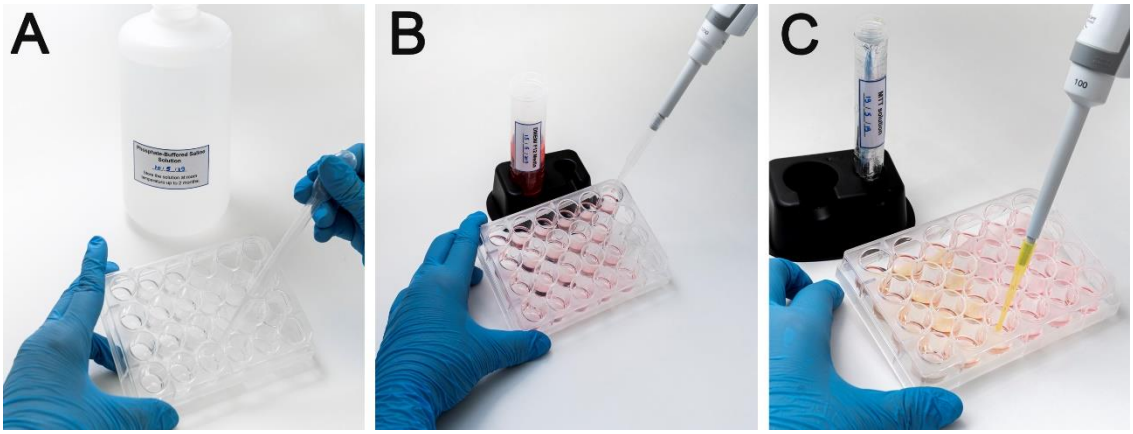

Figure 8. Carefully wash the Sertoli cells with Phosphate buffered saline solution (PBS) at room temperature (A). To each well add the used medium (without Fetal Bovine serum, if is the case) (B), then add the MTT solution (C), both of this solution should be warmed at 37ºC.

4. Add 500 μL of culture medium (without FBS) at 37ºC, to each well (Figure 8, Panel B).
5. Carefully add 50 μL of MTT at 37ºC (Figure 8, Panel C) and carefully shake the plate.
6. Incubate the plate for 2-3 hours at 37ºC (Figure 9, Panel B), protected from the light by wrapping the plate in aluminium foil (Figure 9, Panel A). After the incubation time, carefully remove the medium with the MTT solution without removing the cells (Figure 9, Panel C).

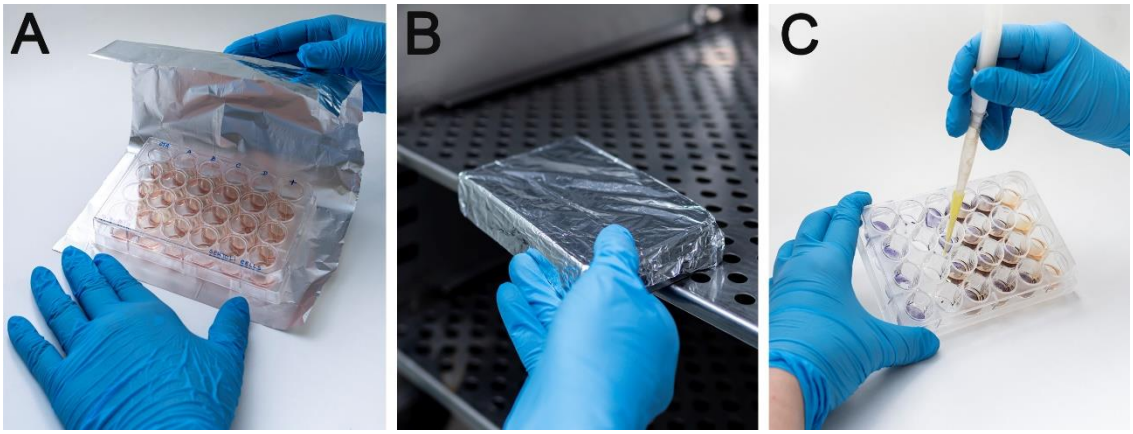

Figure 9. Wrap the plate in aluminum foil to protect from light (A) and incubate for 1-4 hours to 37ºC (B). Carefully remove the medium (C), the formazan crystals are observed in a purple coloration

7. Add 350 μL of DMSO (Figure 10, Panel A) and shake the plate for at least 10 minutes, or until the crystals are solubilized (Figure 10, Panel B).
8. Transfer 100 μL from each well to a 96-wells plate (Figure 10, Panel C) (this should be done in triplicate to validate the readings). Use 100 μL of DMSO for blank. If necessary, dilute the samples in DMSO (Figure 11, Panel A).
9. Read the plates at 570 nm and 650-655 nm in a multiplate reader (Figure 11, Panel B).

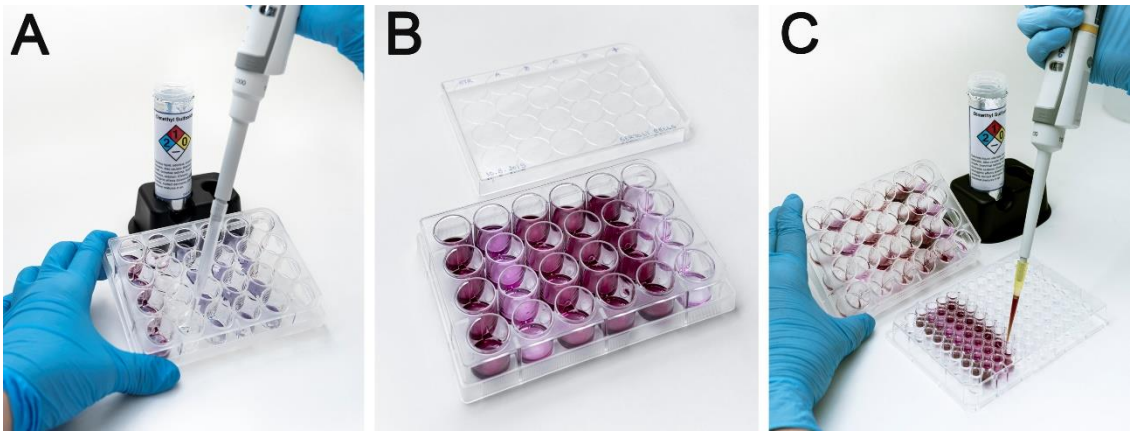

Figure 10. Use DMSO to solubilize the formazan crystals (A), this solubilization originates a purple solution (B) and then transfer the obtained solution from each well to a 96-well plate (C).

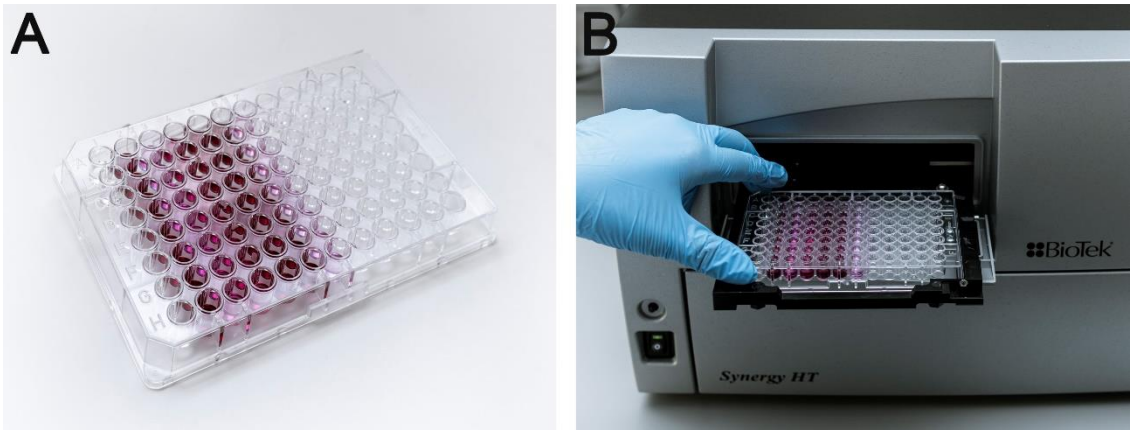

Figure 11. The coloration of the plate should be darker as the compound is less toxic to the cells, DMSO is used as blank (A). Finally, read the optic density in a multi-well plate reader (B)

10. Data treatment: To assess Sertoli cells proliferation after an experimental treatment use the following data treatment:
    I. If you performed the read in triplicates, calculate the average of the values for each experimental condition

II. Subtract the value measured at 570 nm wavelength to the one measured at 650-655 nm in the samples and in the blanks and then subtract the blank value:

$$Abs_{sample\ adjusted} = Abs_{sample\ at\ 570nm} - Abs_{sample\ at\ 650-655\ nm}$$

$$Abs_{sample} = Abs_{sample\ adjusted} - Abs_{blank}$$

III. Assuming the condition without any compound as your control (100%), calculate the proliferation of the other conditions based on the control group:

$$\%\ Cell\ proliferation_{Condition\ A} = \frac{Abs_{Samples\ Condition\ A}}{Abs_{\ Samples\ Control}} \times 100$$

Support Protocol

Establishment of human Sertoli cells primary cultures

Experiments with Sertoli cells can be performed using immortalized cell lines or cells from primary cultures of Sertoli cells derived from human [15-18], rat [19], mouse [20] or other animals [21, 22]. The assessment of Sertoli cell proliferation using MTT and SRB can be performed in any type of Sertoli cell, obtained from primary (independent of the origin) or immortalized cultures. There are some available Sertoli cell lines in the market: 15P-1, TM4 (ATCC®), SER-W3 (DZMS®), among others. In the case of selection of Sertoli cells from immortalized cell lines, the instructions from the manufacturer should be followed to establish and maintain the cell culture. The protocol to isolate primary Sertoli cells of rat, mice and bovine is already well described [2]. To culture primary human Sertoli cells from small testicular biopsies, which is a rare protocol, follow the instructions below.

Material List

- 20G sterile needle
- Syringe sterile
- Plastic Pasteur pipette sterile
- Cell culture flask
- Incubator at 33ºC (5% CO_2)
- Centrifuge at 300.g
- 15 ml conical tube sterile
- Phosphate buffered saline solution sterile (see recipe in Reagents and Solutions)

- Culture media sterile (see recipe in Reagents and Solutions)
- Laminar flow cabinet

Steps and Annotations

1. In a laminar flow cabinet under a sterile environment perform the following steps.
2. If the testicular biopsy is suspended in phosphate buffered saline solution or any other saline solution, centrifuge the conical tube at 300.*g* at room temperature for 5 minutes.
3. Discard the supernatant and with a sterile plastic pipette add 5 mL of phosphate buffered saline solution at 33ºC and resuspend the sample to disaggregate possible cells clusters.
4. Centrifuge the tube with the sample once again at 300.g at room temperature for 5 minutes and at the end discard the supernatant.
5. With a plastic Pasteur pipette add 5 mL culture media and in order to perform a mechanical digestion force the sample to pass thought a 20G needle.
6. Add the appropriate volume to a cell culture flask and then the sample after mechanical digestion. Then shake the flask and place it in the incubator at 33ºC.
7. The culture flask with the cells should not be disturbed during at least 2 days. After that, the state of the culture should be examined under a microscope to access confluence and check for possible contaminations.
8. The culture media should be replaced every other day. To replace the media, under aseptic conditions, discard the media.
9. Wash very carefully three times with phosphate buffered saline solution at 33ºC.
10. Add fresh culture media at 33ºC to the cells and place the flask in the incubator.

Reagents and Solutions (Figure 2, Panel A & 7, Panel A)

Phosphate-buffered saline Solution

- Sodium chloride (NaCl) 8 g
- Potassium chloride (KCl) 0.2 g
- Sodium phosphate dibasic (Na2HPO4) 1.44 g
- Potassium phosphate monobasic anhydrous (KH2PO4) 0.24 g
- Weight all the reagents, dissolve in distilled water to a volume of 1 L and set the pH to 7.4.
- Store the solution at room temperature up to 2 months.

Fixing Solution (1% acetic acid + 99% Methanol)

- Add 5 mL of acetic acid to 495 mL of methanol.
- Store the solution at -20ºC up to 6 months.

0.05% SRB Solution

- Weight 50 mg of SRB and dissolve in to 1% acid acetic solution to a volume of 100 mL.
- Store the solution at room temperature up to 1 year.

1% Acetic Acid Solution

- Add 5 mL of acetic acid to 495 mL of distilled water.
- Store the solution at room temperature up to 6 months.

Tris 10 mM solution (pH=10)

- Weight 60.6 mg of Tris-Base and dissolve in dissolve in distilled water to a final volume of 500 mL and set the pH to 10 using 1 N NaOH.
- Store the solution at room temperature up to 4 months.

MTT solution

- Thiazolyl Blue Tetrazolium Bromide (C18H16BrN5S) 10 mg
- Weight 10 mg of MTT, dissolve in 2 mL of PBS protected from light.
- Make it fresh, store at room temperature until the use.

Culture Media

- The culture media for Sertoli cells is Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM: F12) with 18 mM of glucose, supplemented with 10% of fetal bovine serum and antibiotics (50 μg/mL gentamicin, 50 U/mL penicillin and 50 mg/mL streptomycin) at a pH of 7.4. The culture media should be used sterile.

Commentary

Background Information

These two protocols are just two simple ways to determinate cell proliferation/toxicity but there are some other options available, such as, lactate dehydrogenase, sodium 3′-[1-[(phenylamino)-

carbony]-3, 4-tetrazolium]-bis (4- methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT) or trypan blue. In addition, there is a large offer of commercial kits available for a wide range of prices and protocols. Other commercial kits and the commercial version of the SRB and MTT assays are much more expensive. Other disadvantage that can be considered is the self-time of the commercial kit, that is usually much shorter than the reagents needed to perform the assays needed to set the proposed protocols. Regarding the time necessary to perform the proliferation assays using commercial kits or the proposed protocols, it is similar.

The application of these protocols in Sertoli cells is just a first step to determinate the toxicity of a compound. SRB assay is widely used to assess toxicity in primary cultures of rat [19] and human Sertoli cells [23, 24]. MTT assays is widely used to assess cytotoxicity in mouse Sertoli cells lines [25-27], primary cultures of mouse [26], rat [28] and piglet [29] Sertoli cells. This is just a first screening to assess a mechanistic explanation for the effects, more specific and time-consuming studies must be performed. These protocols are always performed in triplicate and with at least an n equal to 6, considering distinct passages.

MTT assay

The MTT assay is not a real proliferation assay as it can be more accurately considered a metabolic proliferation assay, since it is based on the activity of a dehydrogenase present in the mitochondria. However, it is known that metabolic activity is increased in proliferating cells, and compounds with a toxic effect will decrease this activity.

Critical Parameters and Troubleshooting

In order to correctly determine the proliferation of Sertoli cells is necessary to ensure that each well of the culture plate contains the same amount of cells. For that, make sure that your cell suspension is correctly homogenate at the time of the seeding.

The volumes in this protocol are recommended for a 24-well plate, however it can be adapted to the use of other plates with a different number of wells.

Most of the available protocols for SRB and MTT indicate the use of 96-wells culture plates to seed the cells. In the specific case in discussion, Sertoli cells, we recommend the use of 24-wells (or 48-wells) plates depending of the compound concentrations in study. This option is also ideal to avoid the artefacts of the cells in the bottom of the plates, as is usually known to happen in the case of performing these studies in a 96-well plate.

The confluence of the cells in each plate should be approximately 70%. However, since the evaluation of confluence is usually visually assessed, it can vary a little introducing a factor that

can cause some problems to the reliability of the assay. Please note that this protocol can be applied to Sertoli cells of different origins and thus, each cell type requires different times to achieve confluence, and even in the same cell type the confluence time can vary but one would expect more or less 72 hours. In addition, it is very important to note that a confluence higher than 90% is not viable to perform any of these assays as an overload of cells cause severe perturbations in the readings.

In both protocols, the washing steps and the addition of solutions to the wells where the cells were seeded should be carefully performed once there is a risk of detaching the cells from the plate. Finally, the removal of the solutions from the well plates should also be carefully performed, if possible, with a vacuum bomb with a very thin tip.

MTT Assay

MTT is very toxic, and thus, at the time of weight, the compound must be carefully used with an appropriate individual protection, as indicated by the manufacture of the compound (gloves, tight sealing safety goggles, laboratory coat and handle the reagent in accordance good hygiene and safety practice).

If the optic density (OD) after adding DMSO is too high (above 1), please dilute all the samples from each well, in the same proportion, with DMSO.

Anticipated Results

Since these are colorimetric assays, it is expected a change in coloration depending of the effect of the studied compound in Sertoli cell proliferation. In both protocols it is expected a decrease in the intensity of the coloration with the increase of the compound toxicity and consequently, a decrease in cell proliferation or increase in cell death.

Time Considerations

SRB Assay

This protocol has no need to be performed immediately after two hours of fixation at -20ºC. The fixation must be done for at least 2 hours and after that step the protocol can be performed when desired. Also, the option to dry at room temperature can be extended until necessary or in option it can be done at 37ºC in an incubator as a way to perform the protocol in less time. If you want to do a pause in the protocol at the drying steps, save your plate at room temperature with the cover plate.

MTT Assay

Contrary to SRB protocol, MTT assays must be performed at once and the plate cannot be saved to do it later. The incubation of the plate with MTT requires a minimum time of 2 hours, but it can be extended as some protocols indicate that it can go up until 4 hours.

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Chapter 5

Leptin modulates human Sertoli cells acetate production and glycolytic profile: a novel mechanism of obesity-induced male infertility?

This chapter was adapted from the published work:

Martins AD, Moreira AC, Sá R, Monteiro MP, Sousa M, Carvalho RA, Silva BM, Oliveira PF, Alves MG. Leptin modulates human Sertoli cells acetate production and glycolytic profile: a novel mechanism of obesity-induced male infertility? Biochimica et Biophysica Acta - Molecular Basis of Disease. 1852(9): 1824-1832 (DOI: 10.1016/j.bbadis.2015.06.005)

A.D. Martins collected the data, performed the experiments and analyzed the results. A.D. Martins drafted, edited, contributed and approved the final version the manuscript.

Leptin modulates human Sertoli cells acetate production and glycolytic profile: a novel mechanism of obesity-induced male infertility?

Abstract

Human feeding behavior and lifestyle are gradually being altered, favoring the development of metabolic diseases, particularly type 2 diabetes and obesity. Leptin is produced by the adipose tissue acting as a satiety signal. Its levels have been positively correlated with fat mass and hyperleptinemia has been proposed to negatively affect male reproductive function. Nevertheless, the molecular mechanisms by which this hormone affects male fertility remain unknown. Herein, we hypothesize that leptin acts on human Sertoli cells, the “nurse cells” of spermatogenesis, altering their metabolism. To test our hypothesis, human Sertoli cells were cultured without or with leptin (5, 25 and 50 ng/mL). Leptin receptor was identified by qPCR and Western blot. Protein levels of glucose transporters (GLUT1, GLUT2 and GLUT3), phosphofructokinase, lactate dehydrogenase (LDH) and monocarboxylate transporter 4 (MCT4) were determined by Western Blot. LDH activity was assessed and metabolite production/consumption determined by proton nuclear magnetic resonance. Oxidative damage was evaluated by assessing lipid peroxidation, protein carbonylation and nitration. Our data shows that leptin receptor is expressed in human Sertoli cells. The concentration of leptin found in lean, healthy patients, upregulated GLUT2 protein levels and concentrations of leptin found in lean and obese patients increased LDH activity. Of note, all leptin concentrations decreased human Sertoli cells acetate production illustrating a novel mechanism for this hormone action. Moreover, our data shows that leptin does not induce or protect human Sertoli cells from oxidative damage. We report that this hormone modulates the nutritional support of spermatogenesis, illustrating a novel mechanism that may be linked to obesity-induced male infertility.

Introduction

Human eating habits and lifestyle have been dramatically changing. Increased consumption of energy together with a sedentary lifestyle has led to a positive energy balance. These changes contribute to an increased incidence of obesity and associated metabolic diseases. Until recently, these chronic metabolic diseases were only associated with aging. However, this paradigm is

being shifted and a growing number of children, adolescents and young adults in reproductive age are affected by these pathological conditions [1].

Leptin is a peptide hormone mainly produced in adipose tissue stores [2]. It was initially called "satiety hormone", since it was thought to be solely produced by adipocytes of white adipose tissue, to control energy homeostasis and decrease food intake [3]. More recently, leptin has been reported to be also produced in other tissues [4]. The plasma concentration of leptin tends to be increased in most obese individuals and positively correlated with total body fat [5, 6], with the exception of the rare individuals with congenital leptin deficiency [7]. In fact, leptin possesses anti-obesity functions, based on its ability to suppress appetite and decrease body weight and adiposity [2]. Notably, leptin is now a Food and Drug Administration (FDA) approved [8] therapeutic for several medical conditions.

The biological actions of leptin are carried out through interaction with the specific membrane-spanning leptin (or obesity) receptor (Ob-R) [9]. This hormone also mediates metabolic signals to the reproductive system, denoting when sufficient fat stores are available to meet the caloric demands of the reproductive events [10]. It has been suggested that the hypothalamus may be the primary target for most of leptin's actions on the reproductive axis [11]. However, based on the characterization of leptin receptor distribution and the effects of leptin on *in vitro* systems, direct action sites for this hormone have been suggested both in female [12] and male reproductive tissues [13-15].

Several studies using a leptin deficient rodent model emphasized the role of leptin in male reproductive function. *Ob/ob* mice present an autosomal-recessive mutation on chromosome 6, promoting a profound decrease in circulating leptin levels. Among other characteristics, these mice are obese and infertile [10, 16]. Of note, a low-dosage leptin treatment restored fertility in *ob/ob* male mice [17]. In fact, those leptin-treated mice presented increased testicular and seminal vesicle weight [10, 16, 17] and elevated sperm counts [10]. In humans, congenital leptin deficiency is associated with hypogonadotropic hypogonadism, which may be reverted upon recombinant leptin treatment [18]. Yet, although it is known that leptin crosses the Sertoli-Sertoli cell barrier (BTB) [19] and is present in the seminal plasma [14], the molecular mechanisms by which it regulates male reproductive function remain unknown. The Sertoli cell, which is the somatic component of BTB, plays an essential role in spermatogenesis. These cells are responsible for the physical and nutritional support of the developing germ cells [20]. The preferential substrate of developing germ cells is lactate, which is produced by the Sertoli cell from several metabolic sources, particularly glucose [21]. The metabolic cooperation established between Sertoli cells and developing germ cells is essential for the occurrence of spermatogenesis [20, 22]. The Sertoli

cell metabolism is sensitive to hormonal fluctuations and presents an enormous metabolic plasticity [23]. Therefore, we hypothesized that exposure to leptin can affect spermatogenesis by modulating Sertoli cell metabolism. To test our hypothesis, we firstly evaluated the expression of the Ob-R on human Sertoli cells. Then, human Sertoli cells were exposed to three distinct concentrations of leptin (the physiological concentration found in lean, healthy patients and in seminal plasma; a concentration usually detected in obese patients and a concentration found in morbidly obese individuals). The effects on metabolite production/consumption and protein levels and/or activity of key glucose and monocarboxylate transporters and metabolic enzymes were determined. Finally, since leptin has been suggested to alter oxidative equilibrium in cells, oxidative damage in exposed human Sertoli cells was evaluated by assessing lipid peroxidation, protein carbonylation and nitration.

Material and Methods

Chemicals

NZY M-MuLV Reverse Transcriptase, random hexamer primers, dNTPs, NZTaq 2x Green Master Mix, agarose and DNA ladder were obtained from NZYTech (Lisboa, Portugal). Leptin was obtained from Bachem (Bubendorf, Switzerland). Primers were obtained from STABVIDA (Oeiras, Portugal). All other chemicals were purchased from Sigma–Aldrich (St. Louis, USA), unless stated otherwise.

Sertoli Cells Primary Culture

Clonetics™ human Sertoli cells (MM-HSE-2305) were purchased from Lonza (Walkersville, USA). The human Sertoli cells were thawed following the manufacturer protocol optimized by our group. In brief, the vial with frozen cells was thawed at 33ºC and cells were placed in culture flask with Sertoli culture medium (1:1 mixture of DMEM-Ham's F12, pH 7.4) supplemented with 15 mM HEPES, 50 U/mL penicillin, 50 mg/mL streptomycin sulfate, 0.5 mg/mL fungizone, 50 µg/mL gentamicin and 10% heat inactivated fetal bovine serum (FBS). Cells were incubated at 33ºC in an atmosphere of 6% CO_2. Sertoli cells cultured in the presence of 10% FBS in F12:DMEM remain mitotically active as described [24]. The cells used for all experiments were obtained between the third and eighth passage to ensure reproducibility. Each "n" corresponds to a cell passage and all experiments were performed in triplicate. After 96 h, cultures were examined by phase contrast microscopy and human Sertoli cells culture purity was determined as described [25].

Experimental groups

Cells were allowed to grow until reach 80-85% of confluence and serum-starved before treatment. The culture medium was then replaced by serum-free medium (DMEM: F12 1:1, pH 7.4) supplemented with insulin–transferrin–sodium selenite (ITS medium; final concentration of 10 mg/L; 5.5 mg/L; 6.7 µg/L, respectively). To evaluate the effect of leptin on the glycolytic profile of human Sertoli cells we defined a control group with ITS medium without leptin and three groups supplemented with leptin (5 ng/mL, 25 ng/mL and 50 ng/mL). The concentration of 5 ng/mL was chosen agreeing with the physiological levels found in lean, healthy patients [26] and the concentration found in seminal plasma [14]. The concentration of 25 ng/mL was chosen based on the levels reported in the literature for obese patients [26]. We also found relevant to evaluate the effects of a concentration reported in morbidly obese men (50 ng/mL) [27]. After 24 h of treatment, culture medium was collected. Cells were detached, counted with a Neubauer chamber and collected. Viability was evaluated by the Trypan Blue Exclusion test.

Cytotoxicity Assay

The cytotoxicity of human Sertoli cells to leptin was determined by the colorimetric sulforhodamine B (SRB) assay [28]. In brief, cells were seeded and treated with selected concentrations of leptin. After treatment, cells were washed twice in phosphate buffered saline solution and fixed overnight in 1% acetic acid in methanol. Cells were then incubated with 0.5% (w/v) SRB in 1% of acetic acid for 1 h at 37ºC. The unbound dye was removed by washing with 1% acetic acid solution. Dye bound to cell proteins was extracted with 10 mM Tris solution (pH 10) and the optical densities of the resulting media were determined at 540 nm. No cytotoxicity was observed for the doses of leptin used in this work (data not shown).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The extraction of total RNA (tRNA) from human Sertoli cells was performed using the E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Norcross, USA) as indicated by the manufacturer. tRNA concentration and absorbance ratios (A260/A280) were determined by spectrophotometry (Nanophotometer™, Implen, München, Germany). Human liver tRNA was purchased from AMS Biotechnology (Abingdon, UK). tRNA from human Sertoli cells and human liver was reversely transcribed as described [29]. The resulting complementary DNA (cDNA) was used with exon-exon spanning primers set designed to amplify Ob-R (Forward primer: TCT GGA CTG CTC ACG GTC AT; Reverse primer: ACC CAG CAT TTT CAC GGT TTG), Sox-9 (Forward primer: AGG AAG TCG GTG AAG AAC GG; Reverse primer: AAG TCG ATA GGG GGC TGT CT) and GATA-

4 (Forward primer: CTA GCA GCT TCT GCG CCT GT; Reverse primer: GTG GTT CCG GAA GCT GAT GTA). PCR were carried out as described [30]. Primers' optimal annealing temperature was set to 62ºC to Ob-R, 56ºC to Sox-9 and 58ºC to GATA-4. 35 cycles were required for the exponential amplification phase of fragments (180 bp to Ob-R and GATA-4 and 275 bp to Sox-9). Human liver was used as positive control for Ob-R experiments and cDNA-free sample was used as negative control. Samples were run in 1.5% agarose gel electrophoresis (120 mV, 40 min) and visualized using software Molecular Imager FX Pro Plus MultiImager (BioRad, Hercules, USA) coupled to an image acquisition system (Vilber Lourmat, Marne-la-Vallée, France). The size of the expected products was compared to a DNA ladder.

Western blot

Western Blot was performed as described [20]. In brief, protein samples (50 µg) were fractionated on a 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in a Tris-buffered saline solution with 0.05% Tween 20 containing 5% skimmed dried milk, incubated overnight at 4ºC and then incubated with primary antibodies and the conditions presented in Table 1. Mouse anti-β-actin was used as protein loading control. The immune-reactive proteins were detected separately with secondary antibodies and the conditions presented in Table 1. Membranes were reacted with ECF detection system (GE, Healthcare, Weßling, Germany) and imaged with the BioRad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK). The densities from each band were obtained using the Quantity One Software (Bio-Rad, Hemel Hempstead, UK), according to standard methods.

Determination of lactate dehydrogenase (LDH) activity

LDH activity was determined using a commercial assay kit (Promega, Madison, USA), following the manufacturer's instructions as described [30]. The activities measured were calculated using the molar absorptivity of formazan and expressed in nmol/min/mg of protein.

Measurement of oxidative damages

Protein carbonyl, nitro-tyrosine (NT) and 4-hydroxynonenal (4-HNE) group levels were determined by Slotblot. Protein carbonyl groups were determined as described [31]. To determine NT and 4-HNE protein levels, 2.5 µg of protein was diluted in phosphate buffer saline to a final volume of 100 µL and transferred to PVDF membranes. Membranes were then incubated with primary antibodies and the conditions presented in Table 1. The immune-reactive proteins were detected separately with the respective secondary antibodies and the conditions presented in

Table 1. Membranes were reacted with ECF™ substrate (GE Healthcare, Buckinghamshire, UK). Densities from each band were quantified using Quantity One software (BioRad, Vilber Lourmat, France).

Table 1: List of Antibodies used in this study

Antibody	Source	Molecular Weight (kDa)	Dilution	Vendor	Catalog #
GLUT1	Rabbit	55	1:500	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7903
GLUT3	Goat	48	1:500	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7582
GLUT2	Rabbit	61	1:5000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-9117
PFK-1	Rabbit	85	1:400	Santa Cruz Biotechnology, Heidelberg, Germany	sc-67028
MCT4	Rabbit	43	1:1000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-50329
LDH	Rabbit	37	1:10000	Abcam, Cambridge, MA, USA	ab52488
Ob-R	Goat	100 and 125	1:1000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-1835
β-Actin	Mouse	42	1:5000	Sigma-Aldrich, Roedermark, Germany	A5441
DNP	Rabbit	---	1:5000	Sigma-Aldrich, Roedermark, Germany	D9656
4-HNE	Goat	---	1:5000	EMD Millipore, Temecula, CA, USA	AB5605
Nitro-Tyrosine	Rabbit	---	1:2500	Cell Signaling Technology, Danvers, MA, USA	9691S
Mouse	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3562
Rabbit	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3687
Goat	Rabbit	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A4187

Abbreviations: GLUT1- Glucose transporter 1; GLUT2- Glucose transporter 2; GLUT3- Glucose transporter 3; PFK-1- Phosphofructokinase 1; MCT4- Monocarboxylate transporter 4; LDH- Lactate dehydrogenase; DNP- Dinitrophenyl; Ob-R – Obesity Receptor; 4-HNE- 4-Hydroxynonenal

Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy

^{1}H-NMR spectroscopy was used to determine metabolite concentrations in the extracellular media of human Sertoli cells. Fully relaxed ^{1}H-NMR spectra of extracellular media were obtained at 14.1T, 25°C, using a Bruker Avance 600 MHz spectrometer equipped with a 5mm QXI probe with a z-gradient (Bruker Biospin, Karlsruhe, Germany) using standard methods [32]. Sodium fumarate was used as an internal reference (6.50 ppm) to quantify the following metabolites (multiplet, ppm): lactate (doublet, 1.33); alanine (doublet, 1.45); H1-α glucose (doublet, 5.22); acetate (singlet, 1.9); pyruvate (singlet, 1.35). The relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro NMR spectral analysis program (Acorn,

Livermore, USA). The results are expressed as consumption or production of each metabolite in absolute values of pmol/cell.

Statistical analysis

Statistical differences between experimental groups were assessed by two-way ANOVA, followed by Bonferroni's post-test. All data are shown as mean ± SEM (n = 6 for each condition). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). $p<0.05$ was considered significant.

Results

Leptin receptor (Ob-R) is expressed in human Sertoli Cells

Though the seller made available a specific characterization of the cells, we further confirmed that the purchased human Sertoli cells expressed specific Sertoli cells markers (GATA-4 and Sox-9) (Figure S1). The Ob-R was previously identified in rodent testis by RT-PCR and immunohistochemistry [33]. In human testis this receptor was only identified by immunohistochemistry [15, 34]. For Sertoli cells, the Ob-R was only identified in rodent cells by hybridization *in situ* [35]. Therefore, before analyzing the effect of leptin on human Sertoli cells, we investigated the presence of Ob-R in these cells. Using RT-PCR, we were able to detect a 180 bp amplicon in cultured human Sertoli cells, corresponding to the presence of Ob-R mRNA (Figure 1, Panel A). In addition, using a specific Ob-R antibody, we were able to detect a double staining of approximately 100 kDa and 125 kDa (Figure 1, Panel B) corresponding to the presence of the short and long forms of Ob-R.

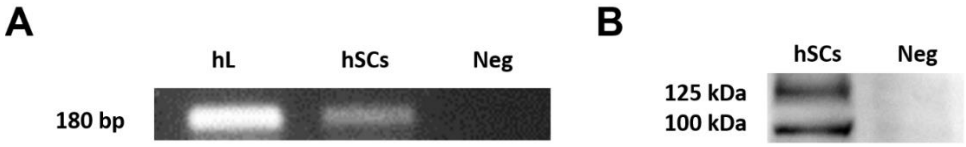

Figure 1: Expression of leptin receptor (Ob-R) in cultured human Sertoli cells (hSCs). Identification of Ob-R by reverse transcriptase polymerase chain reaction (Panel A) and Western Blot (Panel B). hL: human liver; hSCs: human Sertoli cells; Neg: Negative control.

GLUT2 is present in human Sertoli cells and its protein levels are increased after exposure to a concentration of leptin found in lean men

When in culture, human Sertoli cells metabolize glucose to lactate, which is critical for the progression of spermatogenesis. Exposure to leptin was not able to significantly alter the uptake

of glucose by human Sertoli cells (Figure 2, Panel D). The glucose enters in cells through glucose membrane transporters (GLUTs). In human Sertoli cells, two GLUTs isoforms that participate in extracellular glucose uptake have already been identified: GLUT1 and GLUT3 (for review [20]). Exposure to leptin did not alter GLUT1 or GLUT3 protein levels (Figure 2, Panel A and C, respectively). Of note, we were able to identify GLUT2 protein levels for the first time in human Sertoli cells. Using a specific antibody for GLUT2 we detected the specific staining for this protein at 61 kDa (Figure 2, Panel B). In addition, the exposure of human Sertoli cells to the physiological concentration of leptin (5 ng/mL) increased GLUT2 protein levels to 1.13 ± 0.08-fold variation to control (Figure 2, Panel B).

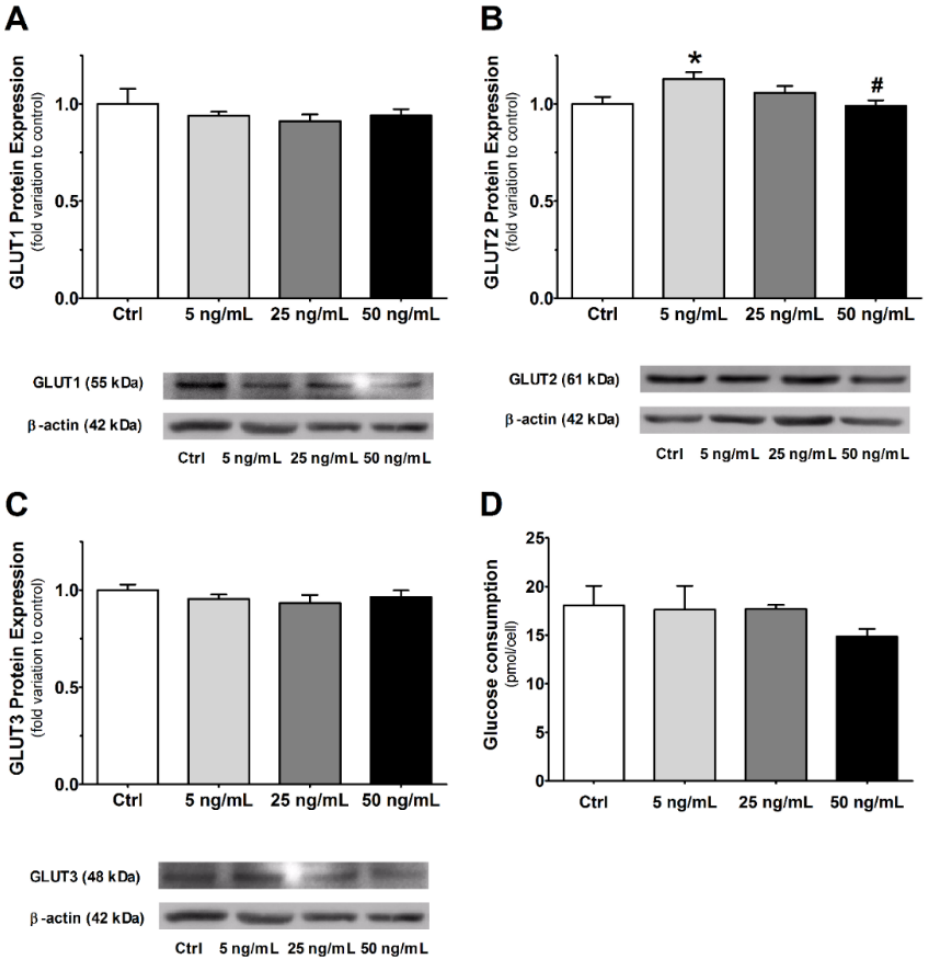

Figure 2: Effect of leptin in glucose transporters and glucose consumption in human Sertoli cells (hSCs). Protein levels of glucose transporter 1 (GLUT1) (Panel A) and glucose transporter 2 (GLUT2) (Panel B) and glucose transporter 3 (GLUT3) (Panel C), as well as glucose consumption (Panel D) by hSCs after exposure to leptin. Panel A-C also show representative Western Blot experiments. Panel A-C shows pooled data of independent experiments, indicating the protein levels of GLUT1, GLUT2 and GLUT3, respectively. Panel D shows pooled data of independent experiments, indicating the consumption of glucose in pmol/cell. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p<0.05$) are as indicated: * relative to control; # relative to 5 ng/mL

Levels of leptin reported in morbidly obese men decreased pyruvate consumption by human Sertoli cells

Glucose is take up by human Sertoli cells through GLUTs and then metabolized. The first rate-limiting step in glucose metabolism is the irreversible conversion of fructose-6-phosphate to fructose-1,6-bisphosphate by phosphofructokinase (PFK). Thus, we analyzed the protein levels of this enzyme. Exposure to all studied leptin concentrations was not able to alter PFK protein levels (Figure 3, Panel A). Pyruvate consumption by human Sertoli cells treated with leptin was found to be decreased only when cells were exposed to a concentration reported in morbidly obese men (Figure 3, Panel B). One of the metabolic pathways involved in the consumption of pyruvate, known to occur in human Sertoli cells, is its conversion to alanine by a reversible reaction catalyzed by alanine aminotransferase. The production of alanine was not altered when these cells were exposed to the different concentrations of leptin used (Figure 3, Panel C) and closely resembled the patterns of pyruvate consumption.

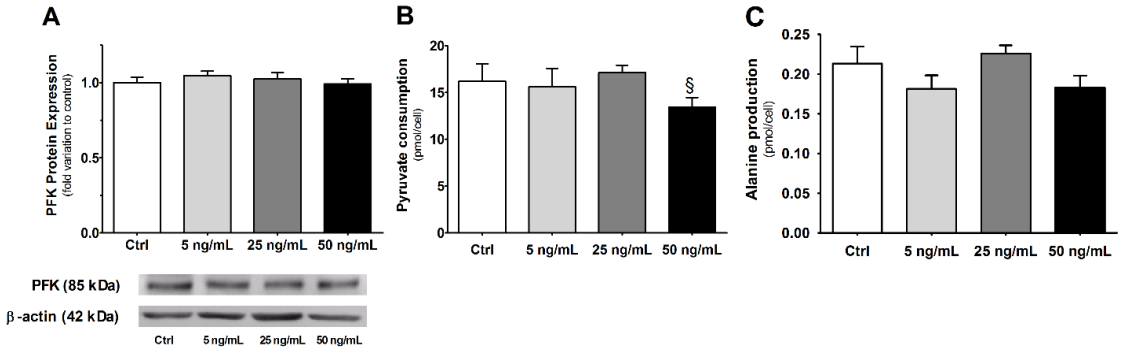

Figure 3: Effect of leptin in glycolysis of human Sertoli cells (hSCs). Protein levels of phosphofructokinase (PFK) (Panel A), as well as pyruvate consumption (Panel B) and alanine production (Panel C) by hSCs after exposure to leptin. Panel A also show representative Western Blot experiments. Panel A shows pooled data of independent experiments, indicating the protein levels of PFK. Panel B-C show pooled data of independent experiments, indicating the consumption of pyruvate and production of alanine in pmol/cell. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($p<0.05$) are as indicated: § relative to 25 ng/mL

Leptin concentrations found in lean and obese men modulate LDH activity

LDH is crucial for the production of lactate by Sertoli cells. LDH protein levels were not altered in human Sertoli cells exposed to increasing doses of leptin (Figure 4, Panel A). However, LDH activity was increased when human Sertoli cells were exposed to leptin concentrations usually found in lean and obese men as compared with cells in control conditions and cells exposed to a concentration of leptin found in morbidly obese patients. The LDH activity increased from 0.64 ± 0.06 nmol/min/mg of protein in control conditions to 1.09 ± 0.14 and 0.83 ± 0.12 nmol/min/mg of

protein when cells were exposed to 5 ng/mL and 25 ng/mL, respectively (Figure 4, Panel B). Furthermore, LDH activity in human Sertoli cells exposed to 5 ng/mL (1.09 ± 0.14 nmol/min/mg) was significantly higher than that of cells exposed to 25 ng/mL (0.83 ± 0.12 nmol/min/mg). Of note, a concentration of leptin reported in morbidly obese individuals decreased LDH activity to 0.50 ± 0.08 nmol/min/mg of protein (Figure 4, Panel B), when compared with cells from all other experimental conditions. Yet, although alterations in LDH activity were observed among human Sertoli cells of the different experimental groups, no differences were detected in the export/production of lactate by human Sertoli cells exposed to the different concentrations of leptin (Figure 4, Panel C and Panel D, respectively).

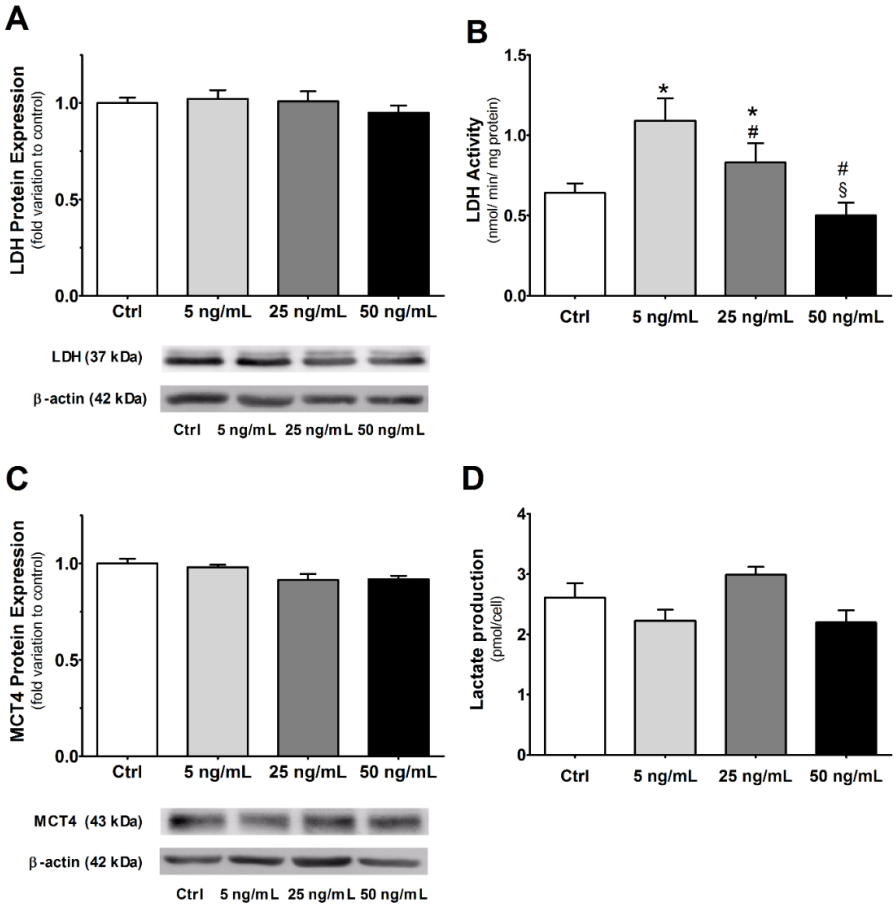

Figure 4: Effect of leptin in lactate metabolism of human Sertoli cells (hSCs). Lactate dehydrogenase (LDH) protein levels (Panel A) and activity (Panel B), as well as, protein levels of monocarboxylate 4 (MCT4) (Panel C) and lactate production (Panel D) by hSCs after exposure to leptin. Panel A and C show pooled data of independent experiments, indicating the protein levels of LDH and MCT4, respectively. Panel B and D show pooled data of independent experiments, indicating the activity of LDH or production of lactate in pmol/cell. Panel A and C also show representative Western Blot experiments. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p<0.05$) are as indicated: * relative to control; # relative to 5 ng/mL § relative to 25 ng/mL

Acetate production by human Sertoli cells is decreased after exposure to leptin and no oxidative damages were detected in the cells after treatment with leptin

Sertoli cells are known as lactate producers, however it was recently reported that they also produce high amounts of acetate [22]. The production of acetate in human Sertoli cells treated with leptin presented a decrease when compared to control condition. The acetate production by human Sertoli cells in control condition was 1.31 ± 054 pmol/cell and 0.46 ± 0.15 pmol/cell, 0.62 ± 0.10 pmol/cell and 0.52 ± 0.15 pmol/cell in cells treated with 5 ng/mL, 25 ng/mL and 50 ng/mL, respectively (Figure 5, Panel B). Pyruvate metabolism, particularly the lactate/alanine ratio, is linked to intracellular redox homeostasis since it reflects the NADH/NAD^+ equilibrium [36]. Exposure to leptin, in any of the concentrations used, does not alter the intracellular redox state of human Sertoli cells (Figure 5, Panel A). The high glycolytic rates detected in human Sertoli cells can lead to increased levels of oxidative stress (OS). Few studies have reported that different leptin levels are associated with variations in OS biomarkers levels [37, 38]. Protein carbonylation/nitration and lipid peroxidation are excellent biomarkers of OS. DNP, 4-HNE and NT are products formed from the attack of free radicals to proteins and membranes. Our data showed that exposure of human Sertoli cells to concentrations of leptin found in lean, obese and morbidly obese men, did not alter carbonyl group (Figure 6, Panel A), NT group (Figure 6, Panel B) nor 4-HNE group (Figure 6, Panel C) levels.

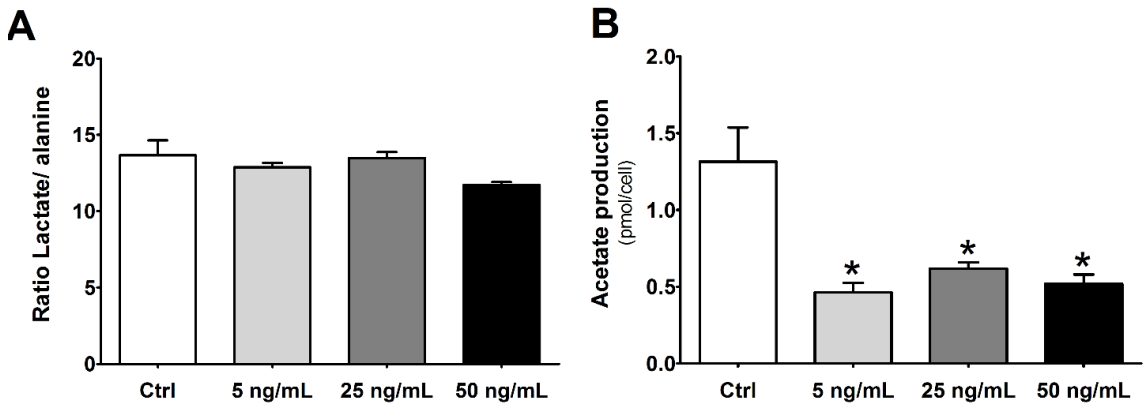

Figure 5. Effect of leptin in acetate production by human Sertoli cells (hSCs) and intracellular redox state. Acetate production (Panel A) by hSCs and lactate/alanine ratio (Panel B) after exposure to leptin. Panel A show pooled data of independent experiments, indicating the production of acetate in pmol/cell and Panel B show pooled data of independent experiments, indicating the lactate/alanine ratio. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p<0.05$) are as indicated: * relative to control.

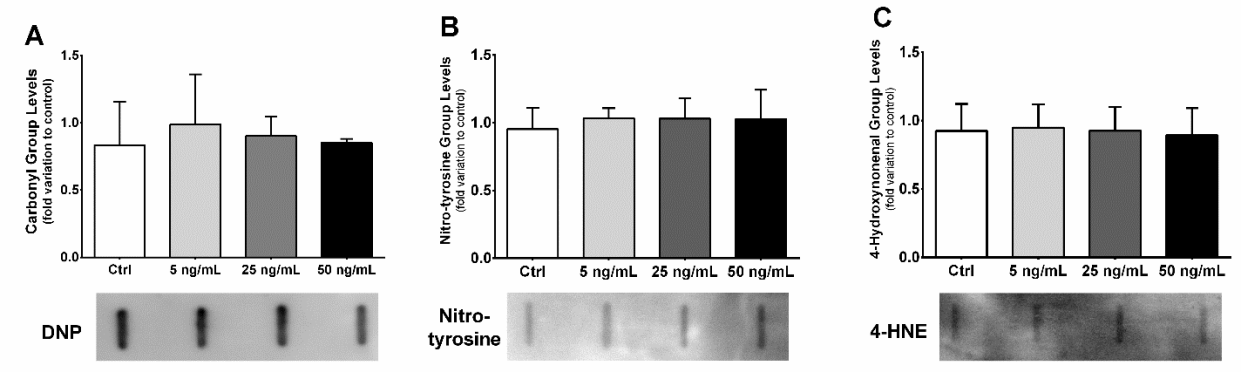

Figure 6. Study of oxidative damages in human Sertoli cells (hSCs) after exposure to leptin. Carbonyl (Panel A), nitro-tyrosine (NT) (Panel B) and 4-hydroxynonenal (4-HNE) (Panel C) group levels measured in hSCs after exposure to leptin. Panel A, B and C show pooled data of independent experiments, indicating the expression levels of carbonyl, NT and 4-HNE, respectively. Panel A, B and C also show representative Slot-blot experiments. Results are expressed as mean ± SEM (n = 6 for each condition).

Discussion

Leptin is crucial in the regulation of body glucose metabolism. Most obese patients present a high level of leptin, which is correlated with their adiposity [39]. Obesity has reached pandemic proportions and the number of obese individuals is expected to dramatically increase in the next decades. One of the negative health consequences associated with obesity in men is reduced fertility [40]. Subfertility/infertility in overweight and obese men has been proposed to be due to suppression and/or alteration of Sertoli cell function [41]. One of Sertoli cells main functions is the metabolic support of spermatogenesis (for review [20]). Since leptin is reported to be involved in the metabolic control of reproduction (for review [42]) and Sertoli cells produce the lactate needed for developing germ cells, we hypothesized that leptin can modulate Sertoli cells metabolism, affecting the normal occurrence of spermatogenesis and altering the fertility potential of males. The action of leptin [43] in male reproductive function has been reported [10, 17] and Ob-R was identified in rodent and porcine testicular and epididymal tissue [13, 44] and Leydig cells [35], while there is no consensus on its presence in Sertoli cell [35, 44]. In humans, the presence of the Ob-R was reported in testicular tissue [15, 34] though the authors reported lack of immunoreactivity inside the tubules and suggested that human Sertoli cells did not express this receptor. Yet, our results attained by PCR and confirmed by Western blot, are the first to clearly demonstrate that Ob-R is expressed in isolated human Sertoli cells.

The Sertoli cells take glucose from circulation and produce metabolites essential to sustain the development of germ cells. Although the presence of GLUT1 has not been observed yet in human testis by immunohistochemistry [45], recent studies using molecular biology techniques [20, 46,

47] reported that extracellular glucose uptake by Sertoli cells is mainly achieved by GLUT1 and GLUT3. This GLUT1/GLUT3 system is very sensitive to hormonal exposure, particularly insulin [22]. GLUT2 expression as already been identified in 42GPA9 cell line immortalized from mouse Sertoli cells by PCR [43] and though expression of GLUT2 was suggested to be non-detectable in human testis by immunohistochemistry [45], we report, for the first time, that cultured human Sertoli cells abundantly express GLUT2, illustrating that this transporter can play a crucial role in glucose transport. Leptin interferes with cellular glucose transport. For instance, it is reported to decrease GLUT3 expression in the hypothalamic neuronal cell line RCA6 [48]. However, in cells with high glycolytic flux, such as breast tumors cells, leptin does not alter GLUT1 expression [49]. In human Sertoli cells, leptin does not alter GLUT1 and GLUT3 protein levels. Nevertheless, GLUT2 protein levels are increased after exposure to a concentration of leptin reported in the plasma of lean men [26]. However, when human Sertoli cells were exposed to the concentration found in the plasma of obese and morbidly obese men, no alteration was observed in GLUT2 protein levels, relatively to control. Evidence shows that this hexose transporter is involved in the control of food intake [50] and, in liver, leptin also up-regulates GLUT2 levels [51]. Although the exact contribution of GLUT2 to the pool of glucose taken by human Sertoli cells from the interstitial fluid remains to be unveiled, the stimulation of GLUT2 protein levels by the concentration of leptin found in lean men, illustrates a crucial mechanism dependent of this hormone. However, glucose consumption by human Sertoli cells was not altered by exposure to leptin. We have previously discussed (for review [23]) that Sertoli cells present a high metabolic plasticity. For instance, when cultured in insulin deprivation conditions [22], or after exposure to endocrine disruptors [52], these cells change the expression of glucose transporters to sustain glucose uptake. Leptin modulates GLUT2 protein levels, illustrating an effect also reported in other highly metabolic systems such as the liver [53]. Notably, the concentration of leptin found in morbidly obese men induced a decrease in GLUT2 protein levels when compared with the concentration detected in lean men providing evidence that this mechanism may be relevant to obesity-related male subfertility/infertility. Nevertheless, further studies will be needed to test this hypothesis and disclose the relevance of this difference in GLUT2 expression.

In Sertoli cells, the vast majority of glucose is metabolized to pyruvate which is then converted to lactate. Although no changes were detected on the protein levels of PFK or LDH, LDH activity was stimulated by the concentration of leptin detected in lean men. Notably, LDH activity decreased by exposure to leptin in a dose-dependent manner, being progressively smaller when human Sertoli cells were exposed to doses observed in obese and morbidly obese men. This suggests that LDH activity may be a key control point of leptin action in spermatogenesis.

However, the observed changes in GLUT2 protein levels and LDH activity did not result in alteration on the production of lactate by human Sertoli cells. This illustrates that although leptin modulates mechanisms associated to lactate production, these cells present a metabolic plasticity that under our experimental conditions allows them to sustain the production of lactate.

Although the lactate produced by Sertoli cells is referred to as the central metabolite for spermatogenesis, these cells synthesize other metabolites essential for the development of germ cells. Germ cells are constantly being duplicated and their proliferation rate is quite high. Human Sertoli cells produce and export high amounts of acetate [22] that is proposed to be essential to maintain the high rate of lipid synthesis in developing germ cells [22], an hypothesis that was not yet tested but likely to occur. Leptin affects lipid catabolism in human placenta [54] and promotes the synthesis of acyl-coenzyme A, a pivotal intermediate in fatty acids metabolism, during monocyte-macrophage differentiation [55]. Moreover, it is reported that acetate may have an inhibitory effect on leptin secretion in epididymal adipocytes [56]. As referred, during spermatogenesis, there is a high rate of lipid synthesis and remodeling [57] and acetate is known to be the most reliable carbon source for lipid synthesis [58]. Acetate production by Sertoli cells is very sensitive to hormonal treatment, particularly insulin [22] and melatonin [59]. Our results show that acetate production by human Sertoli cells is severely decreased after exposure to all concentrations of leptin. This is consistent with reports showing that leptin decreases acetate degradation in isolated adipocytes and its incorporation into lipids [60]. In fact, leptin directly suppresses some biochemical pathways in peripheral tissues and cells, particularly those involving acetyl-CoA carboxylase [61]. Since all leptin concentrations, including the concentration found in lean men, altered acetate production by human Sertoli cells, further studies are needed to explore the hypothesis that this is a relevant mechanism for obesity-related subfertility/infertility by having a synergistic action with other hormones and factors.

Though absolute differences in metabolites concentration detected are due to exposure to leptin and thus reveal the direct action of this hormone in human Sertoli cells, we must highlight that absolute quantifications differ from our previous reports [22, 62, 63]. This difference in absolute quantification is due to several factors, particularly those that might not allow a simple comparison: the ITS supplement is very different; the stoichiometric pressure over some pathways, instead of other is very distinct; and this study was done after 24 hours of culture while the others were performed at least in 48 hours. Moreover, the human Sertoli cells used were purchased and only then expanded while our previous studies were done using human Sertoli cells isolated from fresh biopsies. The results attained allowed us to assess the effect of leptin in human Sertoli cells.

Compelling evidence suggests that leptin's action induces OS and reactive oxygen species (ROS) may serve as second messengers in leptin-mediated signaling. Moreover, it has been reported that leptin induces OS in human endothelial cells (10 ng/ml) [64] and plasma of Wistar rats [65]. The cellular glucose sensing machinery and cell metabolism are closely related to OS, particularly the lactate/alanine ratio [29]. Indeed, this ratio is an index of cellular redox state [66]. The higher is the NADH level in the cytosol, the more extensive is the conversion of pyruvate to lactate by LDH, and such leads to a considerable reduction of the alanine pools through the action of alanine transaminase. Of note, when exposed to some compounds such as the antidiabetic metformin [30], Sertoli cells adapt their metabolic behavior to maintain the NADH/NAD^+ equilibrium. Interesting, in human Sertoli cells, the intracellular redox equilibrium remains unaltered after exposure to leptin. Moreover, the group levels of carbonyl, NT and 4-HNE were not altered after human Sertoli cells were exposed to leptin, illustrating that this hormone does not promote oxidative damage in these cells even at concentration found in morbidly obese men.

Obesity and its associated co-morbidities have reached pandemic proportions. Men suffering with those diseases have fertility problems that end-up in subfertility and infertility. Among the several hormonal and metabolic alterations caused by the expansion of fat mass, increased leptin levels is a well-known characteristic of these individuals. Leptin acts in several tissues and cellular systems altering their metabolic behavior. For instance, leptin was reported to stimulate the metabolic activity of NK-92 cells, which belong to the innate immune system and mediate several anti-tumor responses [67]. Besides, hyperleptinemia is associated with carcinogenesis [68], which is known to be related with an increased glycolytic profile of cells [69]. Within the testis, the glycolytic activity of Sertoli cells is pivotal to the normal occurrence of spermatogenesis. Herein we report that Ob-R mRNA and protein are expressed in human Sertoli cells and that leptin directly acts on these cells modulating their metabolic behavior. Leptin stimulates GLUT2 protein levels and LDH activity, and severely decreases the production of acetate by human Sertoli cells. This is a first assessment of how leptin can interfere with the metabolic support of spermatogenesis by human Sertoli cells. Further studies will be needed to fully disclose the role of leptin in Sertoli-germ cells interaction and its relevance for male fertility.

Supplemental Data

Figure S1: Expression of Sox-9 and GATA-4 in cultured human Sertoli cells (hSCs). Identification of Sox-9 (Panel A) and GATA-4 (Panel B) by reverse transcriptase polymerase chain reaction. hSCs: human Sertoli cells; hL: human liver; Neg: Negative control.

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Chapter 6

Ghrelin acts as energy status sensor of male reproduction by modulating Sertoli cells glycolytic metabolism and mitochondrial bioenergetics

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Ghrelin acts as energy status sensor of male reproduction by modulating Sertoli cells glycolytic metabolism and mitochondrial bioenergetics

Abstract

Ghrelin is a growth hormone-releasing peptide that has been suggested to interfere with spermatogenesis, though the underling mechanisms remain unknown. We studied the effect of ghrelin in human Sertoli cells metabolic phenotype. For that, human Sertoli cells were exposed to increasing concentrations of ghrelin (20, 100 and 500 pM) mimicking the levels reported in obese, normal weight and severely undernourished individuals. The metabolite production/consumption was determined. The protein levels of key glycolysis-related transporters and enzymes were assessed. The lactate dehydrogenase (LDH) activity was measured. Mitochondrial complexes protein levels and mitochondria membrane potential were also determined. We showed that human Sertoli cells express the growth hormone secretagogue receptor. At the concentration present in the plasma of normal weight men, ghrelin caused a decrease of glucose consumption and mitochondrial membrane potential in human Sertoli cells, though LDH activity and lactate production remained unchanged, illustrating an alteration of glycolytic flux efficiency. Exposure of human Sertoli cells to levels of ghrelin found in the plasma of severely undernourished individuals decreased pyruvate consumption and mitochondrial complex III protein expression. All concentrations of ghrelin decreased alanine and acetate production by human Sertoli cells. Notably, the effects of ghrelin levels found in severely undernourished individuals were more pronounced in human Sertoli cells metabolic phenotype highlighting the importance of a proper eating behavior to maintain male reproductive potential. In conclusion, ghrelin acts as an energy status sensor for human Sertoli cells in a dose-dependent manner, showing an inverse association with the production of lactate, thus controlling the nutritional support of spermatogenesis.

Introduction

Regulation of whole-body energy homeostasis is essential for a proper functioning of male reproductive function. This event involves a fine coordination between central regulators and

peripheral nutrient-sensing molecules. In fact, an inverse U-shaped association has been established between body mass index (BMI) and male fertility, in which both obese and underweight men are less likely to have children than normal weight individuals [1]. It has also become evident, by the data available in the literature, that starvation and malnutrition can impair fertility. Diets poor in nutrients and rich in fats and sugars are responsible for the increased incidence of obesity and associated metabolic diseases (e.g. diabetes mellitus or metabolic syndrome). As individuals consume less healthier diets, a concomitant and rapid increase in the number of children, adolescents and young adults diagnosed with metabolic diseases is observed, raising the concerns over the fertility status and reproductive health of male population (for review [2]).

The leptin-ghrelin axis mediates the hormonal control of food intake and energy homeostasis. In particular, ghrelin controls energy metabolism in such a way that it mirrors the energy status of the individual [3, 4]. In fact, negative energy balance (body stores) translates into increased circulating levels of ghrelin, while a surplus energy balance (obesity) is reflected in a decrease in ghrelin levels [5, 6]. In what concerns the male reproductive system, it has been reported that gut hormones may cooperate with other regulatory signals in the integrated control of energy homeostasis and reproduction [7, 8]. Indeed, ghrelin inhibits the proliferative activity of immature Leydig cells *in vivo* [9] and, more recently, it was proposed that it acts as a modulator of spermatogenesis avoiding excess of build-up of germ cells [10]. Ghrelin outcomes in tubular stem cell factor expression was also reported [9], which has been described as crucial for spermatogonia survival [11]. As for the effect of this hormonal axis on Sertoli cells, while it was recently shown that leptin directly modulates glucose metabolism in human Sertoli cells [12], the effects of ghrelin remain unknown. So far, only the presence of its receptor, the growth hormone secretagogue receptor (GHS-R) [13] has been reported in rat and human Sertoli cells, by means of the immunohistochemistry technique [7, 14].

The efficiency of spermatogenesis is intrinsically associated with Sertoli cells population and function [15], and particularly with its metabolism [16]. Notably, there is a relationship between the number of germ cells supported by each Sertoli cell and sperm production [17]. The Sertoli cells are known as “nurse cells” since they are responsible for the physical and nutritional support of developing germ cells [16] and for maintaining the ionic homeostasis of the tubular fluid [18, 19]. In these cells, membrane glucose transporters (GLUTs) are responsible for the uptake of glucose [20], which is primarily metabolized to pyruvate and then converted to lactate by lactate dehydrogenase (LDH) [20]. This metabolite is then exported by monocarboxylate transporter 4 (MCT4) [21] to be used by germ cells for the production of energy, regulating their metabolic

activity and survival [22]. Besides lactate, Sertoli cells also produce other metabolic cofactors and substrates, such as acetate, which is suggested to be crucial for the high rate of lipid synthesis by germ cells [23]. A normal spermatogenesis is highly dependent on the metabolic cooperation established between Sertoli and germ cells, a process known to be sensitive to hormonal regulation [20, 24].

Although there is strong evidence that ghrelin has a central role in male reproduction, the molecular mechanisms by which this hormone controls spermatogenesis remain unknown. We hypothesize that ghrelin can affect spermatogenesis through a direct action on Sertoli cell function, modulating its bioenergetic status, in a manner that may be linked to the nutritional status of the individual. To test our hypothesis, we evaluated the glycolytic and bioenergetic profile of human Sertoli cells in the presence of levels of ghrelin found in obese, normal weight and severely undernourished individuals in order to *in vitro* mimic the situations found in the clinical setting.

Materials and Methods

Chemicals

HEPES was purchased from Acros Organics (Geel, Belgium). Gentamicin was purchased from Lonza (Basel, Switzerland). Heat inactivated fetal bovine serum and Trypsin–EDTA solution were purchased from Biochrom AG (Berlin, Germany). Ghrelin was purchased from Bachem (Weil am Rhein, Germany). Primers were purchased from STABVIDA (Oeiras, Portugal). Agarose and DNA ladder were purchased from NZYTech (Lisboa, Portugal). Sulforhodamine B (SRB) was purchased from Biotium (Hayward, CA, USA). ECF detection system was purchased GE, Healthcare (Weßling, Germany). Human liver total RNA (tRNA) was purchased from AMS Biotechnology (Abingdon, UK). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Patient's selection and testicular tissue preparations

The patient's clinical study and testicular tissue handling was performed at the Centre for Reproductive Genetics Professor Alberto Barros (Porto, Portugal) in accordance with the Guidelines of Local (P.N. 12/12 CES), National and European Ethical Committees. The studies were also performed according to the Declaration of Helsinki. The testicular biopsies used in this study were obtained from patients under treatment for recovery of male gametes who gave informed written consent. Only cells left in the tissue culture plate after patient's treatment were used. Human Sertoli cells were isolated from testicular biopsies of six men with conserved

spermatogenesis (subjected to a previous vasectomy or with psychological, vascular, or neurologic anejaculation). Those patients were submitted to testicular sperm extraction (TESE) under sedation to obtain spermatozoa for intracytoplasmic sperm injection.

Sertoli Cells Culture

Human Sertoli cells were obtained by a method optimized by Oliveira and collaborators [25]. In brief, each testicular biopsy was transferred to sperm preparation medium (SPM) (Medicult, Copenhagen, Denmark) containing 50 U/mL penicillin and 50 mg/mL streptomycin until cell isolation. The biopsies were then digested in a solution of SPM containing DNase and collagenase IV. Then, testicular cells were washed in $HBSS_f$ (Hanks Balanced Salt Solution without Ca^{2+} or Mg^{2+}) through centrifugations at 300.g at room temperature as previously described [26]. $HBSS_f$ was added and the suspension passed through a glass Pasteur pipette, and pelleted at 300.g for 5 min. This procedure was repeated twice. The resultant cellular pellet was suspended in Sertoli cells culture medium (DMEM: Ham's F12, 1:1, containing 15 mM HEPES, 50 U/mL penicillin and 50 mg/mL streptomycin sulfate, 0.5 mg/mL fungizone, 50 µg/mL gentamicin and 10% heat inactivated FBS) and forced through a 20G needle, in order to disaggregate large cell clusters. Then, cells were plated on cell culture flasks (VWR, Carnaxide, Portugal) and incubated at 37°C, 6% CO_2. After 96 h, cultures were examined by phase contrast microscopy and only those with cell contaminants below 5% were used. Sertoli cell culture purity was tested by the immunoperoxidase technique. In brief, we used an ExtrAvidin Peroxidase Staining Kit (Sigma-Aldrich, St. Louis, MO, USA) in which the cells were incubated with primary polyclonal antibody and processed by the labelled streptavidin–biotin method [27] (Figure S1).

Experimental design

Cells were allowed to grow until 90-95% of confluence, and then washed. The culture medium was replaced by serum-free medium (DMEM: F12 1:1, pH 7.4) supplemented with insulin-transferrin-sodium selenite (ITS) medium (final concentration of 10 mg/L; 5.5 mg/L; 6.7 µg/L, respectively). To evaluate the effect of ghrelin on the glycolytic profile of Sertoli cells, four different groups were defined: a control group without ghrelin and three groups containing ITS medium supplemented with ghrelin (20 pM, 100 pM and 500 pM). The concentration of 100 pM was selected based on levels found in normal weight healthy individuals [28, 29]. The concentration of ghrelin 20 pM, was chosen based on levels that are often reported in obese individuals [6, 29]. We also evaluated the effects of exposure to an elevated ghrelin concentration (500 pM) as that reported in individuals under states of severe malnutrition [30, 31]. After 24 h treatment, culture

medium was collected, and the cells detached from the flask using a Trypsin–EDTA solution. Viability was evaluated by the Trypan Blue Exclusion test, averaging 85–90%. Then, cells were counted with a Neubauer chamber and collected for protein extraction.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Quantitative real-time PCR (qPCR)

Extraction of tRNA from human Sertoli cells and human adipose tissue was performed using the E.Z.N.A. tRNA Kit (Omega Bio-Tek, Norcross, USA) as indicated by the manufacturer. RNA concentrations were determined by Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). tRNA was reversely transcribed as previously described [32]. The resultant complementary DNA (cDNA) was used with exon-exon spanning primer sets designed to amplify GHS-R (forward: TCT GGA CTG CTC ACG GTC AT; reverse: ACC CAG CAT TTT CAC GGT TTG); GATA4 (forward: CTA GCA GCT TCT GCG CCT GT; reverse: GTG GTT CCG GAA GCT GAT GTA) and SOX9 (forward: AGG AAG TCG GTG AAG AAC GG; reverse: AAG TCG ATA GGG GGC TGT CT) cDNA fragments. PCR were carried out as previously described [32]. Primer's optimal annealing temperature of 62ºC, 58ºC, 56ºC, respectively, and 35 cycles were required for exponential amplification phase of fragments. The fragments size was 180 bp and cDNA of human adipose tissue was used as positive control sample for GHS-R. GATA4, and Sox 9 fragments size were 189 bp and 275 bp, respectively, and cDNA of human liver was used as positive control sample. cDNA-free sample and RT-free cDNA were used as negative control. Samples were run in 1.5% agarose gel electrophoresis and visualized using the software Molecular Imager FX Pro Plus MultiImager (Biorad, Hercules, CA, USA) coupled to an image acquisition system (Vilber Lourmat, Marne-la-Vallée, France). The size of the expected products was compared to a DNA ladder. qPCR was performed to analyse the mRNA expression levels of GHS-R in the different experimental groups. qPCR experiments were carried out in a StepOne™ (Applied Biosystems, Foster City, CA, USA) and efficiency of the amplification was determined for all primer sets using serial dilutions of cDNA as described [33]. Amplification conditions were followed as described [34] for GHS-R and with an annealing temperature of 60ºC to β-2-microglobulin (forward: ATG AGT ATG CCT GCC GTG TG; reverse: CAA ACC TCC ATG ATG CTG CTT AC). Transcript levels were used to normalize the mRNA expression of GHS-R. The fold variation the target gene expression was calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta Ct}$ [35].

Cytotoxicity Assay

The cytotoxicity of ghrelin on human Sertoli cells was evaluated by the colorimetric SRB assay [36], as described by Jesus [37]. In brief, cells were seeded and treated with the selected concentrations of ghrelin used in this study. After treatment, cells were washed twice in phosphate buffered saline solution and fixed overnight in 1% acetic acid in methanol. Cells were then incubated with 0.5% (w/v) SRB in 1% of acetic acid for 1 h at 37ºC. The unbound dye was removed by washing with 1% acetic acid solution. Dye bound to cell proteins was extracted with 10 mM Tris solution (pH 10) and optical densities of the resulting media were determined at 540 nm. No cytotoxicity was observed for the concentrations of ghrelin used in this study (data not show).

Western blot

Western Blot was performed as previously described [23]. In brief, proteins samples (50 µg) were fractionated on a SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in a Tris-buffered saline solution (TBS) with 0.05% Tween 20 containing 5% skimmed dried milk and incubated overnight at 4ºC with primary antibodies (see Table 1). Mouse β-actin was used as protein loading control. The immune-reactive proteins were separately detected with secondary antibodies (see Table 1). Membranes were reacted with ECF detection system and read with the BioRad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK). The densities from each band were obtained using the Quantity One Software (Bio-Rad, Hemel Hempstead, UK), according to standard methods.

Determination of lactate dehydrogenase (LDH) activity

LDH activity was determined using a commercial assay kit (Promega, Madison, WI, USA) and following the manufacturer's instructions, as previously described [38]. The attained activities were calculated using the molar absorptivity of formazan (19900 $M^{-1}.cm^{-1}$) and expressed in nmol/min/mg of protein.

Nuclear Magnetic Resonance (NMR) spectroscopy

^{1}H-NMR spectroscopy was used to determine metabolite concentrations in the extracellular media of human Sertoli cells as previously described [39]. Sodium fumarate was used as an internal reference (6.50 ppm) to quantify the following metabolites (multiplet, ppm): lactate (doublet, 1.33); alanine (doublet, 1.45); H1-α glucose (doublet, 5.22); acetate (singlet, 1.9); pyruvate (singlet,

1.35). The relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro™ NMR spectral analysis program (Acorn, Fremont, CA, USA).

Table 1: Antibodies used in this study

Antibody	Source	Molecular Weight (kDa)	Dilution	Vendor	Catalog #
GLUT1	Rabbit	55	1:200	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7903
GLUT2	Rabbit	61	1:5000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-9117
GLUT3	Goat	48	1:100	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7582
PFK-1	Rabbit	85	1:400	Santa Cruz Biotechnology, Heidelberg, Germany	sc-67028
PKM1/2	Rabbit	60	1:1000	Cell Signaling Technology, Danvers, MA, USA	3190
PDH	Rabbit	43	1:1000	Cell Signaling Technology, Danvers, MA, USA	3205
GAPDH	Rabbit	37	1:1000	Cell Signaling Technology, Danvers, MA, USA	5174
HADHB	Mouse	47	1:5000	Abcam, Cambridge, United Kingdom	ab110301
MCT4	Rabbit	43	1:1000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-50329
LDH	Rabbit	37	1:10000	Abcam, Cambridge, UJ	ab52488
Total OXPHOS	Mouse	20, 30, 40, 48, 50	1:1000	Abcam, Cambridge, UK	ab110413
β-Actin	Mouse	42	1:5000	Thermo Fisher, Rockford, IL, USA	MA5-15739
Mouse	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3562
Rabbit	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3687
Goat	Rabbit	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A4187

Abbreviations: GLUT1- Glucose transporter 1; GLUT2- Glucose transporter 2; GLUT3- Glucose transporter 3; PFK-1- Phosphofructokinase 1; PKM1/2 - Pyruvate kinase M1/2 isoform; PDH -pyruvate dehydrogenase; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; HADHB - mitochondrial trifunctional protein subunit beta; MCT4- Monocarboxylate transporter 4; LDH- Lactate dehydrogenase;

Mitochondrial membrane potential

Alterations in mitochondrial membrane potential were evaluated using the cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) dye (Molecular Probes, Eugene, OR, USA). In brief, cells were treated with 1.5 µM JC-1 dye (diluted in DMEM: Ham's F12 plus 1% FBS) for 30 min at 37ºC. In healthy mitochondria, JC-1 forms aggregates observed at excitation wavelength 535 nm and emission wavelength 595 nm. While, in unhealthy mitochondria, JC-1 monomers were observed at excitation wavelength 485 nm and emission

wavelength 530 nm, using a Cytation^3 Imaging Reader (BioTek Instruments, VT, USA). The ratio of the fluorescent intensity of the JC-1 aggregates to fluorescent intensity of monomers was used as an indicator of mitochondria health.

Statistical analysis

Statistical significance among the experimental groups was assessed by two-way ANOVA, followed by Turkey's multiple comparisons test. All data are shown as mean ± SEM (n = 6 for each condition). The D'Agostino & Pearson omnibus normality test was used to determine the normal distribution of the data. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

Results

Growth hormone secretagogue receptor is expressed in human Sertoli cells and its expression increased in cells exposed to levels of ghrelin found in normal weight men

Our results showed that the cells significantly expressed the specific markers GATA4 and SOX9 (Figure S2). GHS-R was previously identified in Sertoli cells by immunohistochemistry in human testicular tissue [14]. Therefore, before analyzing the effect of ghrelin on human Sertoli cells, we investigated the presence of GHS-R in these cells by molecular biology techniques. Using RT-PCR, we were able to detect a 180 bp amplicon in cultured human Sertoli cells, corresponding to the presence of GHS-R mRNA (Figure 1, Panel A). Additionally, we analyzed the mRNA expression of GHS-R in human Sertoli cells exposed to the increasing concentrations of ghrelin by qPCR. When exposed to 100 pM of ghrelin, human Sertoli cells significantly increased the expression of GHS-R (14.74 ± 4.13-fold variation to control) (Figure 1, Panel B).

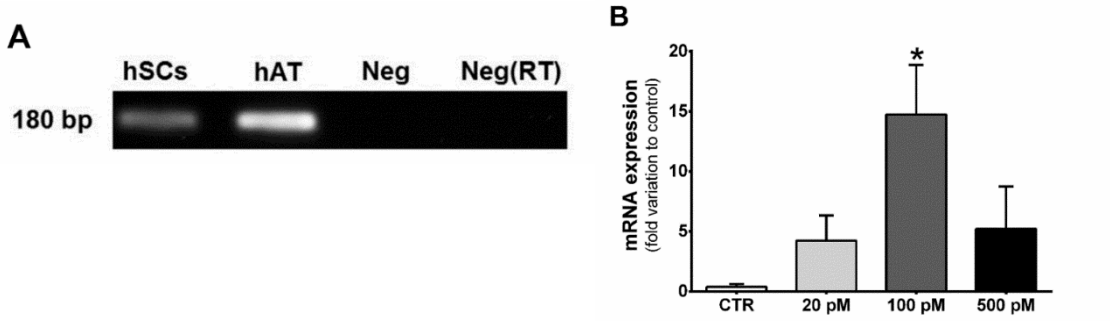

Figure 1: Effect of ghrelin in growth hormone secretagogue receptor (GHS-R) in human Sertoli cells (hSCs). Expression of GHS-R in cultured hSCs by reverse transcriptase polymerase chain reaction (Panel A) hAT: human adiposity tissue; hSCs: human Sertoli cells; Neg: Negative control; Neg (RT): Negative control without reverse transcriptase. Expression of mRNA transcript levels of GHS-R in hSCs after exposure to ghrelin (Panel B). Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are as indicated: * relative to control.

Glucose consumption was decreased, while pyruvate kinase M1/2 isoform protein expression was increased in human Sertoli cells exposed to levels of ghrelin found in normal weight men

In human Sertoli cells, the glycolytic flux is reported to be high [40] and GLUTs are responsible for glucose input to the cell. Exposure of human Sertoli cells to 500 pM ghrelin increased GLUT1 protein levels (1.20 ± 0.09-fold variation to control), which was also increased relatively to the levels found after exposure to 20 pM ghrelin (Figure 2, Panel A). No differences were detected in GLUT2 or GLUT3 protein levels in human Sertoli cells after treatment with any of the ghrelin concentrations studied. Interestingly, human Sertoli cells treated with 100 pM of ghrelin showed a decreased glucose consumption to 12.95 ± 1.55 pmol/cell (Figure 2, Panel D), while cells treated with 20 or 500 pM ghrelin consumed 15.88 ± 3.57 pmol/cell and 16.61 ± 1.71 pmol/cell of glucose, respectively (Figure 2, Panel D). A major rate-limiting step of glycolysis is the reaction catalyzed by phosphofructokinase-1 (PFK-1). Nevertheless, human Sertoli cells treated with ghrelin did not present any significant alteration in PFK-1 protein levels (Figure 3, Panel A). At the end of glycolysis, the produced pyruvate forms phosphoenolpyruvate, in a reaction catalyzed by pyruvate kinase. When human Sertoli cells were exposed to 100 pM of ghrelin, the pyruvate kinase M1/2 isoform (PKM1/2) protein expression was increased to 1.19±0.01-fold variation relative to control (Figure 3, Panel B).

Alanine production by human Sertoli cells was sensitive to ghrelin treatment, while pyruvate consumption was decreased by exposure to levels of ghrelin found in normal weight and severely undernourished individuals

Pyruvate consumption in human Sertoli cells treated with 100 or 500 pM of ghrelin was reduced to 19.8 ± 3.0 pmol/cell and to 20.2 ± 2.7 pmol/cell, respectively, as compared with cells cultured without ghrelin (26.2 ± 3.5 pmol/cell) (Figure 3, Panel C). Alanine production is one of the major pathways where pyruvate can be consumed. In the absence of ghrelin, human Sertoli cells produced 1.2 ± 0.2 pmol/cell of alanine. When human Sertoli cells were exposed to the different concentrations of ghrelin, alanine production decreased to 0.7 ± 0.1 pmol/cell (in cells treated with 20 pM ghrelin) or to 0.7 ± 0.2 pmol/cell (in cells treated with 100 pM ghrelin). Notably, when human Sertoli cells were treated with 500 pM ghrelin, alanine production further decreased to 0.4 ± 0.1 pmol/cell (Figure 5, Panel C). No changes were observed after exposure of human Sertoli cells to ghrelin in protein expression of mitochondrial trifunctional protein subunit beta, glyceraldehyde-3-phosphate dehydrogenase and pyruvate dehydrogenase (Figure S3).

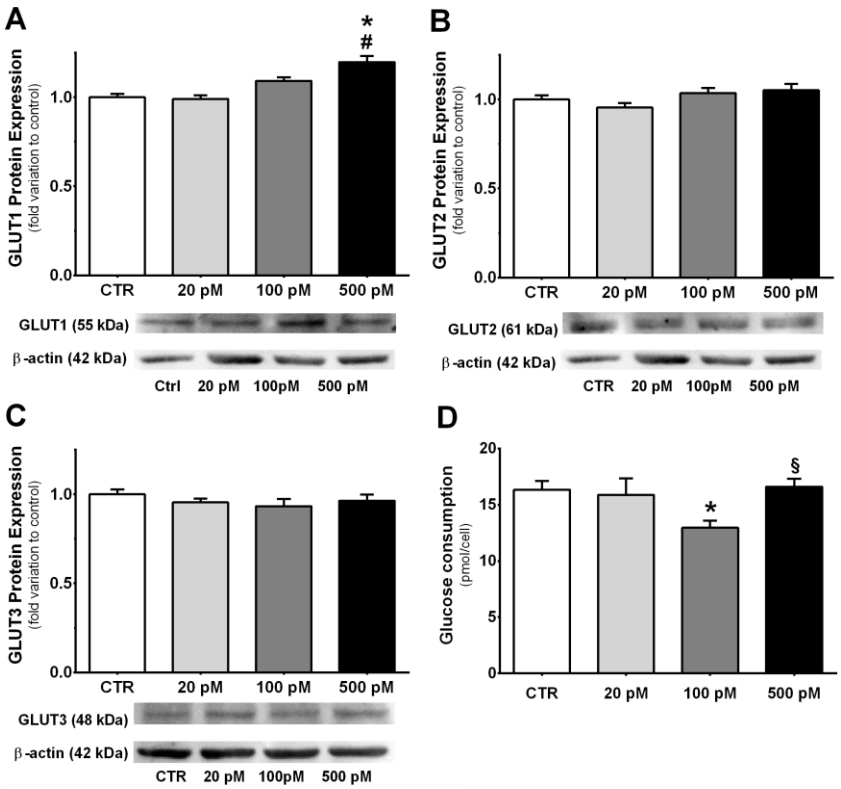

Figure 2: Effect of ghrelin in glucose transporters and glucose consumption in human Sertoli cells (hSCs). Expression of glucose transporter 1 (GLUT1) (Panel A) and glucose transporter 2 (GLUT2) (Panel B) and glucose transporter 3 (GLUT3) (Panel C), as well as glucose consumption (Panel D) by hSCs after exposure to ghrelin are represented. Panels A-C also show representative Western Blot experiments. Panels A-C shows pooled data of independent experiments, indicating the protein levels of GLUT1, GLUT2 and GLUT3, respectively. Panel D shows pooled data of independent experiments, indicating the consumption of glucose. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are as indicated: * relative to control; # relative to 20 pM; § relative to 100 pM.

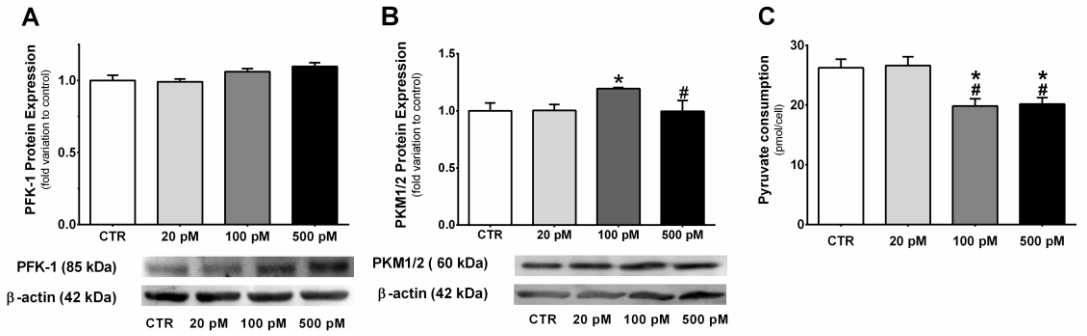

Figure 3. Effect of ghrelin in pyruvate metabolism of human Sertoli cells (hSCs). Expression of phosphofructokinase-1 (PFK-1) (Panel A) and pyruvate kinase M1/2 isoform (PKM1/2) (Panel B), as well as pyruvate consumption (Panel C) by hSCs after exposure to ghrelin. Panel A and B also show a representative Western Blot experiment. Panel A and B shows pooled data of independent experiments, indicating the protein levels of PFK-1 and PKM1/2, respectively. Panel C shows pooled data of independent experiments, indicating the consumption of pyruvate. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p<0.05$) are as indicated: * relative to control; # relative to 20 pM

Treatment of human Sertoli cells with levels of ghrelin described in severely undernourished individuals compromised lactate production

LDH is critical for lactate production by Sertoli cells, which is a recognized pivotal function of these cells. Our data showed that when human Sertoli cells were exposed to ghrelin levels described in severely undernourished individuals, LDH protein expression was increased to 1.23 ± 0.21-fold variation to control (Figure 4, Panel A). Interestingly, LDH activity was decreased from 16.20 ± 1.45 nmol/min/mg of protein in cells cultured without ghrelin to 8.28 ± 1.50 nmol/min/mg of protein in cells cultured with 20 pM ghrelin and to 7.40 ± 0.40 nmol/min/mg of protein in cells cultured with 500 pM ghrelin (Figure 4, Panel B). Only cells exposed to 100 pM ghrelin presented similar values of LDH activity (14.87 ± 1.57 nmol/min/mg of protein) to non-exposed human Sertoli cells (Figure 4, Panel B). The lactate produced by LDH is exported from human Sertoli cells by MCT4. No differences were identified concerning MCT4 protein levels in human Sertoli cells treated with different concentrations of ghrelin (Figure 4, Panel C). Still, lactate production by human Sertoli cells exposed to 500 pM ghrelin (7.88 ± 2.69 pmol/cell) was lower than that observed after cells were treated with 20 pM ghrelin (12.32 ± 2.31 pmol/cell) (Figure 5, Panel A).

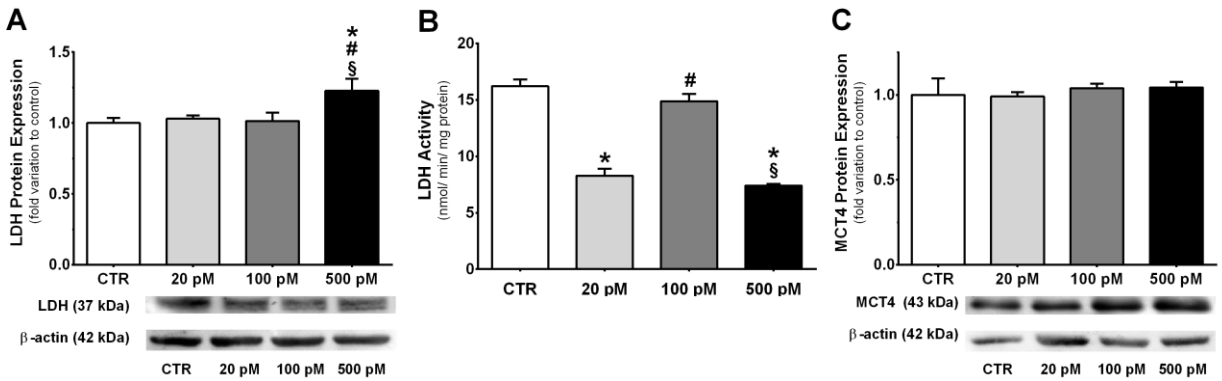

Figure 4: Effect of ghrelin in lactate metabolism of human Sertoli cells (hSCs). Lactate dehydrogenase (LDH) expression (Panel A) and activity (Panel B), as well as, expression of monocarboxylate 4 (MCT4) (Panel C) by hSCs after exposure to ghrelin. Panel A and C show pooled data of independent experiments, indicating the protein levels of LDH and MCT4, respectively. Panel B shows pooled data of independent experiments, indicating LDH activity. Panel A and C also show representative Western Blot experiments. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p<0.05$) are as indicated: * relative to control; # relative to 20 pM and § relative to 100 pM

Exposure to levels of ghrelin described in normal weight and severely undernourished individuals decreased acetate production

Sertoli cells are known as lactate producers but it has been consistently reported that they also produce acetate at high rates [23]. Non-exposed human Sertoli cells produced 1.01 ± 0.17 pmol/cell of acetate and, when exposed to a concentration of 20 pM ghrelin, human Sertoli cells

produced 0.91 ± 0.07 pmol/cell of acetate. Notably, when human Sertoli cells were exposed to 100 or 500 pM ghrelin, they produced less acetate (0.75 ± 0.09 and 0.62 ± 0.12 pmol/cell, respectively) (Figure 5, Panel B).

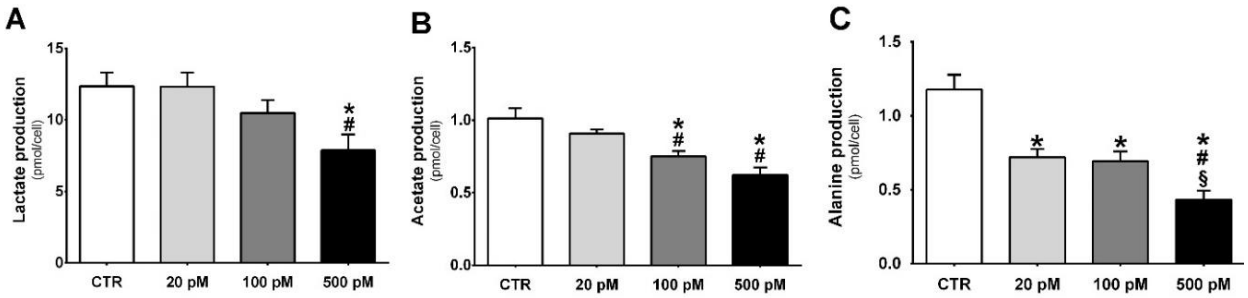

Figure 5: Effect of ghrelin in lactate, acetate and alanine production by human Sertoli cells (hSCs). Lactate (Panel A), acetate (Panel B) and alanine (Panel C) production by hSCs after exposure to ghrelin. Panel A-C show pooled data of independent experiments, indicating the production of lactate, acetate and alanine, respectively. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p<0.05$) are as indicated: * relative to control; # relative to 20 pM and § relative to 100 pM.

Treatment of human Sertoli cells with ghrelin concentration found in normal weight men decreased mitochondrial membrane potential

Our data provided clear evidence that ghrelin modulates mitochondrial complexes protein levels in human Sertoli cells. The mitochondrial complex I protein levels were decreased in cells treated with 500 pM ghrelin to 0.71 ± 0.21-fold variation to control, when compared to non-exposed cells and those treated with 20 pM ghrelin (1.01±0.10-fold variation to control) (Figure 6, Panel A). Protein levels of mitochondrial complex III were increased in human Sertoli cells exposed to 100 and 500 pM ghrelin (1.40±0.18 and 1.33 ± 0.24-fold variation to control, respectively) when compared to non-exposed cells and those treated with 20 pM ghrelin (Figure 6, Panel A). The protein levels of mitochondrial complex V were decreased in human Sertoli cells treated with 20 pM ghrelin found to 0.73 ± 0.08-fold variation relative to control group (Figure 6, Panel A). Human Sertoli cells treated with 100 and 500 pM ghrelin showed increased protein levels of mitochondrial complex V (1.16 ± 0.14 and 1.08 ± 0.16-fold variation to control, respectively) when compared to human Sertoli cells treated with 20 pM ghrelin (Figure 6, Panel A). Nevertheless, our data showed that mitochondrial membrane potential was only decreased in cells exposed to 100 pM ghrelin (concentration found in normal weight men) (JC-1 ratio of 0.64 ± 0.04), when compared to non-exposed hSCs (JC-1 ratio of 0.95 ± 0.09). hSCs treated with 500 pM ghrelin presented a JC-1 ratio of 1.04 ± 0.18, which was increased relatively to human Sertoli cells treated with 100 pM ghrelin (Figure 6, Panel C).

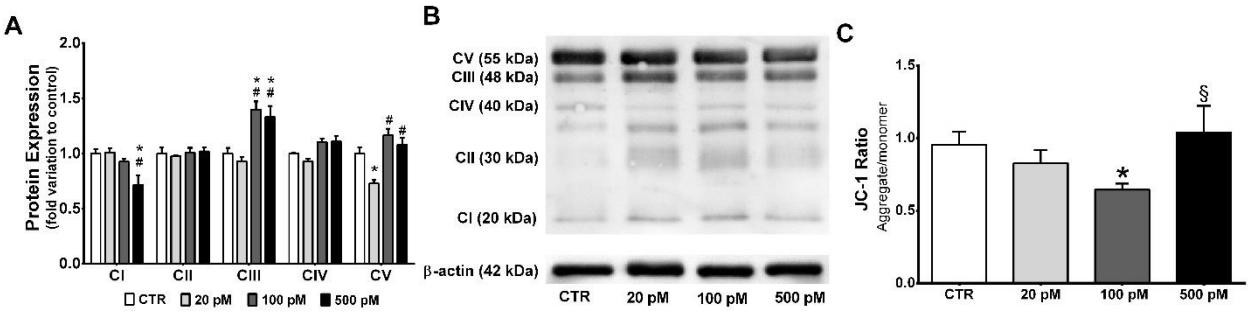

Figure 6: Effect of ghrelin in mitochondria functionality of human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating oxidative phosphorylation (OXPHOS) protein levels (Panel A), its representative Western Blot experiments (Panel B) and JC-1 ratio (Panel C) found in hSCs cultured with or without ghrelin. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($P < 0.05$) are indicated as: * relative to control; # relative to 20 pM and § relative to 100 pM

Discussion

Ghrelin acts as fine-tuned energy status sensor. It plays a key role in metabolic homeostasis by interacting with other central and peripheral regulators of energy expenditure (for review [41]). Moreover, ghrelin seems to modulate whole-body glucose metabolism, and it is involved in the regulation of both glucose tolerance and insulin sensitivity in men and in rodents [42-44]. Ghrelin has a pivotal role in the regulation of metabolic functions. It is closely involved with the development of feeding disorders, such as obesity [45] and some ghrelin effects are mediated by its action in the control of appetite (for review [41]). In fact, ghrelin plasma levels are inversely correlated with the individual's BMI, ranging from values as low as 20 pM in obese individuals to levels above 500 pM in severely undernourished individuals [5, 6, 30, 31].

Reduced fertility is one of the negative health consequences associated with both obesity and low body weight [1, 46]. While on the one hand, alterations and/or suppression of Sertoli cell function in overweight/obese men have been associated with subfertility/infertility [47], undernutrition has also been found to affect testicular structure, reducing Sertoli cell number in the adult [48]. Interestingly, short-term ghrelin administration was reported to decrease the severity of cisplatin-induced gonadal toxicity in mice [49] and suggested to serve as a modulator of testicular apoptosis and proliferation in rats [10]. Moreover, Leydig cells were suggested to act as a paracrine factor in the production of ghrelin and thus, participate in the regulation of spermatogenesis in humans [9, 50]. Catak and collaborators [51] demonstrate, that in rats the levels of ghrelin present in the serum and in the testis are identical. They also suggested that most of ghrelin present in the semen could be a product of the seminal vesicle where the levels

of this hormone are much higher. Although the relevance of ghrelin in reproductive health is implicit, its role in Sertoli cells function, and consequently in spermatogenesis, has not yet been reported.

Spermatogenesis is highly dependent on testicular metabolism [16] and dysregulation of those pathways may lead to subfertility/infertility [32]. The Sertoli cell is responsible for the nutritional support of germ cells, converting glucose to lactate, the preferred substrate of developing germ cells [40, 52], in a process that is tightly modulated by hormones [20, 53]. Herein, we hypothesized that ghrelin may act as energy sensor for male reproductive potential and spermatogenesis by altering human Sertoli cells glycolytic and bioenergetic profiles. Up until now, GHS-R had only been identified in rat testis [54, 55] and rat Sertoli cells [56]. In humans, the presence of ghrelin has only been reported in testicular tissue [57] and in Sertoli cells by immunohistochemistry [14]. Thus, this is the first work providing clear evidence that GHS-R is expressed in isolated human Sertoli cells, by RT-PCR and qPCR, suggesting a role for ghrelin in these testicular cells. Interestingly, when rats are exposed to chronic administration of ghrelin (1 nmol) the number of Sertoli cells were severely decreased [58]. However, our results suggest that when human Sertoli cells are exposed to the concentration of ghrelin found in normal weight men (100 pM) the mRNA expression of GHS-R is enhanced, eliciting a more pronounced effect of the hormone at this concentration.

Spermatogenesis is sustained by Sertoli cells uptaking glucose from circulation to produce various metabolites needed by developing germ cells [16]. GLUT1, GLUT2 and GLUT3 have been proposed to mediate extracellular uptake of glucose by Sertoli cells [12, 26, 59]. Previous studies from our team showed that GLUTs expression in Sertoli cells, particularly that of GLUT1, is very sensitive to hormonal levels, namely of 5α-dihydrotestosterone [60] and insulin [24], with putative consequences on sperm quality. It was also reported that ghrelin is able to stimulate the relative abundance of GLUT1 transcripts in blastocysts at a concentration near the one found in normal weight men (112 pM) [61]. In fact, our data show that only the exposure to ghrelin levels found in severely undernourished individuals caused an increase of GLUT1 expression in human Sertoli cells. In contrast, no alteration was observed for GLUT2 (which is known to be involved in the control of nutrient intake in gastric mucosal cells) [62] or GLUT3 protein levels after exposure of human Sertoli cells to the various doses of ghrelin.

It has been demonstrated that this hormone can alter whole body glucose uptake, affecting also its metabolism. While on the one hand, wild-type mice treated with ghrelin inhibitor presented lower blood glucose levels [63], GHS-R knockout mice fed with a high-energy diet presented augmented glucose uptake relative to wild type mice [64]. Interestingly, our results indicate that

glucose consumption by human Sertoli cells is sensitive to ghrelin concentrations. While human Sertoli cells treated with ghrelin levels found in normal weight men exhibited decreased glucose consumption, cells exposed to the highest dose of ghrelin reversed this effect. The augmented GLUT1 expression observed in human Sertoli cells exposed to ghrelin levels found in severely undernourished individuals might be responsible for the increase in glucose consumption observed in those cells. Still, PFK-1 (a key regulator of the glycolytic pathway) levels were not altered by exposure to any of the concentrations of ghrelin. Another important enzyme is PKM1/2 since it catalyzes the last step of the glycolytic partway. Notably, cells expose to ghrelin 100 pM present an increase in the protein levels of PKM1/2. This could be an adaptive mechanism, in order to sustain the production of lactate in response to a decreased consumption of glucose. Interestingly, when rainbow trout was injected with 100 µg/kg of ghrelin the pyruvate kinase in the hypothalamus also presented an increased activity [65]. These results suggest that pyruvate kinase is under ghrelin control and is sensitive to its concentrations.

Nevertheless, when we considered the consumption by human Sertoli cells of the endpoint metabolite of glycolysis (pyruvate), we could observe a decrease in cells exposed to the two highest concentrations of ghrelin, compared with that expose to levels found in obese individuals. Both the pyruvate taken up from the extracellular medium as that formed at the end of glycolysis can be converted to lactate by LDH. It has been reported that administration of ghrelin (50 µg/kg) in rat hepatocytes after ischemia/reperfusion decreases LDH levels [66], suggesting that this hormone can alter cellular glycolytic flux by modulating LDH expression. Our data showed that exposure of human Sertoli cells to highest levels of ghrelin resulted in a decrease of lactate production, although there was an increase in LDH levels. This may be due to impact of both, the decrease in LDH activity and the lower pyruvate consumption observed in these cells, which illustrates that high levels of ghrelin may alter the metabolic support of spermatogenesis and male reproductive potential by decreasing lactate production.

Besides producing lactate, pyruvate can also enter mitochondria and originate acetyl-CoA to fuel the Krebs cycle. The simultaneous decrease on pyruvate consumption and on alanine and lactate production by human Sertoli cells when exposed to ghrelin levels found in normal weight and severely undernourished individuals, suggests that ghrelin may modulate the Krebs cycle dynamics in these cells. Indeed, several authors have reported that ghrelin causes alterations on mitochondrial function [67-69]. Our results showed that the expression levels of proteins from the mitochondrial complexes were mostly affected when human Sertoli cells were exposed to ghrelin levels found in normal weight and severely undernourished individuals. While the exposure to ghrelin levels found in normal weight and severely undernourished individuals caused an increase

of mitochondrial complex III protein levels, human Sertoli cells decreased the protein levels of mitochondrial complex V when incubated in the presence of lower levels of ghrelin. These results illustrate that protein expression of different mitochondrial complexes is dependent on ghrelin concentration and may be closely related with alterations on mitochondrial functioning. Actually, synaptosomes from wild-type mice subjected to intraperitoneal injection of ghrelin presented a decrease in mitochondrial membrane potential, while those obtained from uncoupling protein 2 knockout mice ($UCP2^{-/-}$) exposed to the same concentration of ghrelin, showed an increase in mitochondrial respiration [70]. Our results show that only incubation with the concentration of ghrelin found in normal weight men caused a decrease in mitochondrial membrane potential, which suggests that this hormone may be involved in the regulation of the mitochondrial function in human Sertoli cells.

Moreover, it has been suggested that acetyl-CoA originated in mitochondria of human Sertoli cells may produce acetate, which is then exported in high quantities by the cells. This process, which may be related with the elevated proliferation rates of developing germ cells [23], is finely tuned by several hormones, particularly by insulin [23], melatonin [71] and leptin [12]. Indeed, acetate is an intermediary for the *de novo* synthesis of fatty acids and cholesterol and thus, it may be crucial for maintaining the high rate of lipogenesis necessary for germ cells development [23]. Several studies highlighted a role for ghrelin in the synthesis of fatty acids and cholesterol. For instance, stimulation of white adipose tissue from mice with 1 nM of ghrelin promotes lipogenesis [72], while ghrelin (10 μM) was reported to downregulate the expression of acyl-coenzyme A (an important intermediate in the metabolism of fatty acids) during monocyte-macrophage differentiation [73]. In our work, when cells were treated with ghrelin levels found in normal weight and severely undernourished individuals the acetate production was decreased, illustrating that ghrelin levels are pivotal for this process in human Sertoli cells.

Hence, we report that GHS-R mRNA is expressed in human Sertoli cells and ghrelin acts directly on these cells modulating their metabolic behavior. Human Sertoli cells exposed to ghrelin levels found in normal weight individuals were able to sustain lactate production, while consuming less glucose and pyruvate than those exposed to ghrelin levels found in obese men. This seemed to be due to the activation of LDH and at the expense of lower production of alanine and acetate, and ultimately of mitochondrial function. On the other hand, human Sertoli cells exposed to ghrelin levels found in obese men were still able to produce similar amounts of lactate, but not as efficiently, for they needed (as referred previously) to consume higher amounts of both, glucose and pyruvate. These cells seem to be redirecting their metabolism towards the Krebs cycle, with a surplus production of acetate. An apparently similar behavior was observed when human Sertoli

cells were exposed to ghrelin levels found in severely undernourished individuals, which in this case seem to exhibit a prioritization of the oxidative metabolism at the expense not only of the production of lactate (due to a sharp decrease of LDH activity), but also of alanine and acetate. Still, these cells consumed higher amounts of glucose than cells exposed to ghrelin levels found in normal weight men, which may represent a failed compensatory mechanism to counteract the lower production of lactate.

In conclusion, ghrelin seems to act as an energy status sensor for human Sertoli cells in a dose-dependent manner, showing an inverse association with the production of lactate, in which Sertoli cells exposed to the ghrelin levels found in severely undernourished individuals are more affected in their function as metabolic supporters of spermatogenesis [1]. When ghrelin is found in low levels, such as in obese men (abundance of nutrients) leads to an increase of pyruvate consumption, shifting to a more oxidative profile, still maintaining the proper lactate production. When ghrelin is found in high levels, such as in severely undernourished individuals, also signals a shift towards an oxidative metabolism (possibly as a strategy to secure human Sertoli cells own energetic needs), compromising the production of lactate. Still, further experiments are needed to clarify the effects of ghrelin in male reproductive health, namely on the metabolic cooperation between Sertoli cell and germ cells and to determine if the effects seen *in vitro* translate to *in vivo* conditions.

Supplemental data

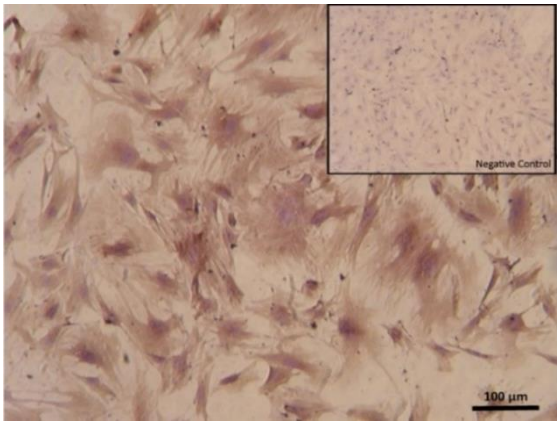

Figure S1: Immunochemical localization of anti-vimentin in cultured human Sertoli cells (hSCs). Purity of the hSCs cultures was assessed by immunostaining with ExtrAvidin Peroxidase Staining Kit. Negative control obtained by omission of the primary antibody (insert panel). Magnification indicated as scale bar.

Figure S2: Expression of GATA 4 (Panel A) and SOX9 (Panel B) in cultured human Sertoli cells (hSCs) by reverse transcriptase polymerase chain reaction (Panel A-B) hL: human Liver; hSCs: human Sertoli cells; Neg: Negative control;

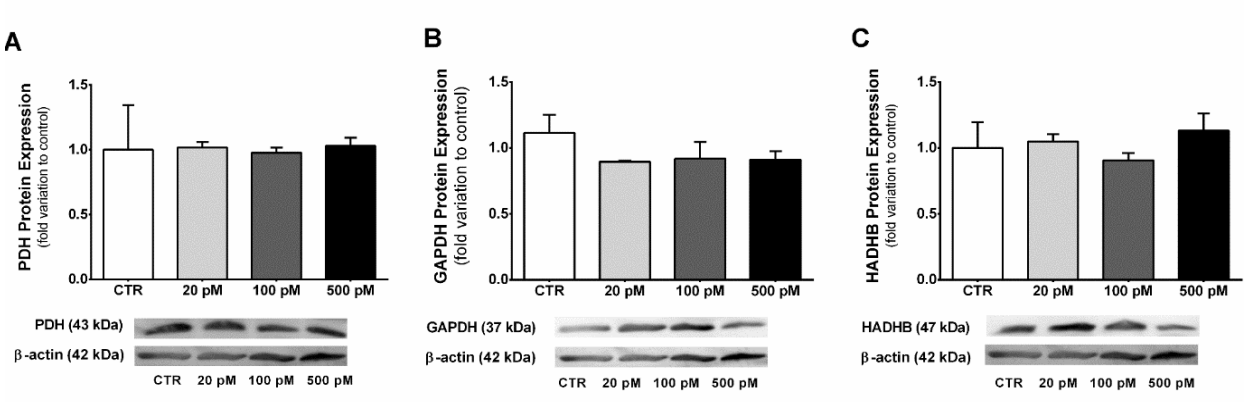

Figure S3: Effect of ghrelin in protein expression of crucial metabolic enzymes in human Sertoli cells (hSCs). Pyruvate dehydrogenase (PDH) (Panel A), glyceraldehyde-3-phosphate dehydrogenase (GAPHD) (Panel B), as well as, mitochondrial trifunctional protein subunit beta (HADHB) (Panel C) protein expression by hSCs after exposure to ghrelin. Panel A -C show pooled data of independent experiments, indicating the protein levels of PDH, GAPDH and HADHB, respectively. Panel A -C also show representative Western Blot experiments. Results are expressed as mean ± SEM (n = 6 for each condition).

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Chapter 7

Metabolic dynamics of human Sertoli cells are differentially modulated by physiological and pharmacological concentrations of GLP-1

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A.D. Martins collected the data, performed the experiments and analyzed the results. A.D. Martins drafted, edited, contributed and approved the final version the manuscript.

Metabolic dynamics of human Sertoli cells are differentially modulated by physiological and pharmacological concentrations of GLP-1

Abstract

Obesity incidence has pandemic proportions and is expected to increase even further. Glucagon-like peptide-1 (GLP-1) based therapies are well-established pharmacological resources for obesity treatment. GLP-1 regulates energy and glucose homeostasis, which are also crucial for spermatogenesis. Herein, we studied the GLP-1 effects in human Sertoli cells metabolism and mitochondrial function. Human Sertoli cells were cultured in absence or exposed to increasing doses of GLP-1 mimicking physiological post-prandial (0.01 nM) levels or equivalent to pharmacological levels (1 and 100 nM) used for obesity treatment. We identified GLP-1 receptor in human Sertoli cells. Consumption/production of extracellular metabolites were assessed, as well as protein levels or activities of glycolysis-related enzymes and transporters. Mitochondrial membrane potential and oxidative damage were evaluated. Glucose consumption decreased, while lactate production increased in human Sertoli cells exposed to 0.01 and 1 nM GLP-1. Though lactate dehydrogenase (LDH) protein decreased after exposure to 100 nM GLP-1 its activity increased in human Sertoli cells exposed to the same concentration of GLP-1. Mitochondrial membrane potential decreased in human Sertoli cells exposed to 100 nM of GLP-1, while formation of carbonyl groups was decreased in those cells. Those effects were followed by an increase in p-mammalian target of rapamycin (mTOR) Ser(2448). Overall, the lowest concentrations of GLP-1 increased the efficiency of glucose conversion to lactate, while GLP-1 concentration of 100 nM induces mTOR phosphorylation, decreases mitochondrial membrane potential and oxidative damage. GLP-1 regulates testicular energy homeostasis and pharmacological use of GLP-1 analogues could be valuable to counteract the negative impact of obesity in male reproductive function.

Introduction

Obesity has emerged as a major healthcare problem increasing the risk of cardiovascular diseases and decreases lifespan [1]. Reduced fertility is a silent obesity-related complication,

which among other co-morbidities is becoming a matter of concern [2]. Dysregulation in the hormonal control of energy homeostasis promoted by the balance between food intake and energy expenditure has been hypothesised to interfere not only in body metabolism, but also with fertility though the underlying mechanisms remain unknown.

Glucagon like peptide-1 (GLP-1) is a 29-amino acid amidated peptide hormone, produced by posttranslational cleavage of the pro-glucagon precursor protein [3]. This peptide is mainly produced by intestinal L-cells and secreted post-prandially [4]. GLP-1 is known for its role in glucose homeostasis predominantly mediated by the incretin effect. It potentiates insulin secretion after oral glucose ingestion, as compared to intra-venous glucose administration. GLP-1 also acts in the central nervous system to reduce the appetite and delays gastric emptying. Glucose equilibrium is crucial for energy homeostasis and the proper functioning of physiological functions, including male fertility. However, due to *in vivo* fast-proteolytic digestion mainly by dipeptidyl peptidase 4 (DPP4) and renal clearance, GLP-1 has a short half-life [5] that limits the use of the native peptide for pharmacological purposes. Thus, several GLP-1 analogues are available, being options for treatment of diabetes and obesity, as these promote weight loss, while having a low risk of causing hypoglycaemia [6].

GLP-1 exerts its activity via GLP-1 receptor (GLP-1R), which belongs to a G-protein coupled receptor family [7]. GLP-1R expression within the male reproductive system has been detected in mice Sertoli cells [8], evidencing that GLP-1 should have an impact on male fertility. In fact, GLP-1R knockout mice have reduced seminal vesicle and gonadal weighs, despite depicting normal testicular sex steroids levels and retaining fertility [9]. While it is known that GLP-1 and its analogues are key regulators of metabolism, the molecular means by which they impact cell metabolism, remain unknown. It has been postulated that mitochondria are the main metabolic targets for this hormone (for review [2]). For instance, GLP-1 improves mitochondrial membrane potential [10] and also mitochondrial mass in a pancreatic cell line [11]. Indeed, both GLP-1 analogues and DPP4 inhibitors, which increase endogenous GLP-1 levels by preventing its degradation, modulate mitochondrial structure and functioning (for review [12]). These metabolic processes depend on signalling pathways network. The mammalian target of rapamycin (mTOR) kinase emerged as pivotal for these processes (for review [13]), controlling mitochondrial bioenergetics and glucose metabolism. GLP-1 interferes with mTOR pathway [14] and recently it was suggested that mTOR has an essential role in male reproduction [15]. In addition, mTOR controls glucose consumption and redox balance of human Sertoli cells [16]. The Sertoli cells are responsible for the nutritional support of spermatogenesis and the major hormonal target inside the testis [17, 18]. Herein, we studied the GLP-1 effects on human Sertoli cells metabolism and

mitochondrial function. We hypothesized that GLP-1 could interfere in human Sertoli cell function with potential implications for the nutritional support of spermatogenesis.

Methods

Chemicals

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise.

Patient's selection and testicular tissue preparation

Testicular tissue was obtained from testicular biopsies (n=6) performed to men with conserved spermatogenesis, suffering from anejaculation due to previous vasectomy or traumatic section of the vas deferens, with the aim of recovering gametes for medically assisted procreation. After informed written consent, human Sertoli cells were isolated from the testicular tissue left in culture plate once gamete retrieval was completed. Testicular biopsies and handling of testicular tissue was done at the Centre for Reproductive Genetics Professor Alberto Barros (Porto, Portugal) in accordance with the Guidelines of Local, National and European Ethical Committees and performed in agreement with the Declaration of Helsinki.

Sertoli Cells Culture

Human Sertoli cells were isolated following the protocol optimized by Oliveira and collaborators [19]. Anti-Mullerian hormone and vimentin, were used, as specific protein markers, to assess the purity of the human Sertoli cells cultures [20]. Only cultures with a purity above 95% were used, Sertoli cells cultures purity was determined by phase contrast microscopy using an ExtrAvidin Peroxidase Staining Kit. To establish the various experimental groups, cells were washed with PBS, and placed in culture serum-free medium (DMEM: Ham's F12, pH 7.4) with insulin-transferrin-sodium selenite medium (final concentration of 10 mg/L; 5.5 mg/L; 6.7 µg/L, respectively) to which GLP-1 (Bachem AG, Bubendorf, Switzerland) was added (or not). One group of cells was not exposed to GLP-1 (0 nM) (no GLP-1) and three other groups were treated with increasing concentrations of GLP-1 (0.01, 1 and 100 nM). The concentration of 0.01 nM was chosen taking into consideration the postprandial GLP-1 levels found in healthy individuals [21, 22]. The other GLP-1 concentrations were chosen to mimic the highest plasmatic concentrations attained after administration of a GLP-1 analogue at the maximum therapeutic dosage recommended for obese individuals (3 mg/daily for liraglutide) either after a single administration (1 nM) or at steady state after a daily administration for 5 weeks (100 nM) [23-25]. After 6 hours of treatment, cell culture medium was collected. We chose this time period for the exposure of

cells to GLP-1 based on GLP-1 half-life [5]. The cells were then washed with PBS and detached using a Trypsin-EDTA solution (0.05% / 0.02% (w/v)), counted using a Neubauer chamber and collected for protein, DNA and RNA extraction. Only groups with viability averaging 85-90%, evaluated by Trypan Blue Exclusion test, were considered for analysis.

RNA and DNA extraction

Total RNA (RNAt) was extracted using the E.Z.N.A.® RNAt commercial Kit (Omega Bio-Tek, Norcross, USA) and DNA was extracted using the E.Z.N.A.® Tissue DNA Kit Commercial Kit (Omega Bio-Tek, Norcross, USA), as indicated by the manufacturer. The amount of DNA and RNA was determinate using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The 260/280 nm of the samples were used to assess the purity of DNA and RNA. DNA presented a ratio of ≈1.8 and RNA presented a ratio of ≈2 in the extracted samples. To assess the integrity of RNA and DNA, an aliquot of each sample was run on a denaturing agarose gel stained with GreenSafe (NZYTech, Lisboa, Portugal).

cDNA synthesis and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNAt was reversely transcribed as previously described [17]. The resultant cDNA was used with exon-exon spanning primer sets designed to amplify specific cDNA fragments (Table 1). PCR was performed with a primer's optimal annealing temperature, using standard methods [17] (Table 1). The samples were visualized using the software Image Lab (BioRad, Hercules, CA, USA) coupled to an image acquisition system BioRad FX-Pro-plus (BioRad, Hemel Hempstead, UK). The product size was compared to a DNA ladder (NZYTech, Lisboa, Portugal). Human heart and human liver RNAt (AMS Biotechnology, Abingdon, UK) were used as positive control and cDNA-free sample was used as negative control.

The mRNA expression levels of GLP-1R were evaluated in the different experimental groups. qPCR experiments were carried out in triplicate, in a CFX 96 qPCR setup (BioRad, Hercules, CA, USA). The efficiency of the amplification for all primers sets was determined by using serial dilutions of cDNA [17]. Amplification conditions were followed as previously described [18]. β-2-microglobulin (β2M) transcript levels were used to normalize the mRNA expression of the target genes. The target genes, sequences and annealing temperatures of the primers are described in Table 1. Following the mathematical model proposed by Pfaffl in the formula: $2^{-\Delta Ct}$, the fold variation the target gene expression was calculated.

Table 1: Primers used in this study

Gene	Primer sequence (5′-3′)	AT(°C)	Amplicon size (bp)
GLP-1R	Sense: GCTCTCCTTCACCTCCTTCC Anti-Sense: TGTCTCTCCCACCTGGATTG	58	300
ND1	Sense: CGATTCCGCTACGACCAACT Anti-Sense: AGGTTTGAGGGGGAATGCTG	60	121
β2M	Sense: ATGAGTATGCCTGCCGTGTG Anti-sense: CAAACCTCCATGATGCTGCTTAC	60	150
$\beta 2M_{nc}$	Sense: GAGGCTATCCAGCGTGAGTC Anti-sense: GACGCTTATCGACGCCCTAA	60	306

Abbreviations: β2M: Beta-2-Microglobulin; $\beta 2M_{nc}$: nuclear encoded beta-2-microglobulin; GLP-1R: Glucagon like peptide-1 receptor; ND1: NADH dehydrogenase, subunit 1.

Determination of mtDNA Copy Number

A qPCR analysis was performed to study the mtDNA copy number as described [26] with small modifications. The efficiency of the amplification was determined by serial dilutions of DNA, and the amplification conditions used were as previously described [27]. The reaction mixture consisted in NZY qPCR Mix (NZYTech, Lisboa, Portugal), primers (Table 1) and 20 ng of mtDNA. Each reaction was carried out in an CFX 96 (Biorad, Hercules, USA). C_t value differences between NADH dehydrogenase subunit 1 (ND1) gene and nuclear encoded beta-2-microglobulin ($\beta 2M_{nc}$) gene were used to quantify mtDNA copy number relative with the following model proposed by Pfaffl: $2^{-\Delta Ct}$.

Cytotoxicity Assay

A sulforhodamine B (SRB) colorimetric assay was performed to test the cytotoxicity of GLP-1 to human Sertoli cells [28]. The cells were seed and treated with the selected concentrations of GLP-1. After 6 hours, the assay was performed as previously described [17]. No cytotoxicity was observed for GLP-1 concentrations used (data not shown).

Western blot

Total proteins isolated from human Sertoli cells were extracted using the M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, USA). Western blot was performed as previously described [27]. The membranes were incubated overnight at 4ºC with primary and secondary antibodies (Table 2). Mouse β-tubulin was used as protein loading control. ECF

detection system was used and the membranes were read in the BioRad FX-Pro-plus (BioRad, Hemel Hempstead, UK). The densities from each band were quantified according to standard methods using Image Lab (BioRad, Hemel Hempstead, UK). Whenever possible the membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, USA) following the manufacturer's instructions, blocked and marked with other primary and consequently secondary antibody.

Table 2: Antibodies used in this study

Antibody	Source	Molecular Weight (kDa)	Dilution	Vendor	Catalog #
GLUT1	Rabbit	55	1:500	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7903
GLUT2	Rabbit	61	1:5000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-9117
GLUT3	Goat	48	1:500	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7582
MCT4	Rabbit	43	1:1000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-50329
LDH	Rabbit	37	1:10000	Abcam, Cambridge, MA, USA	ab52488
Catalase	Mouse	60	1:4000	Sigma-Aldrich, Roedermark, Germany	C0979
P-mTOR	Rabbit	289	1:500	Cell Signaling Technology, Danvers, MA, USA	2971S
β-Tubulin	Mouse	50	1:5000	Thermo Fisher Scientific, Rockford, USA	MA5-16308
DNP	Rabbit	---	1:5000	Sigma-Aldrich, Roedermark, Germany	D9656
4-HNE	Goat	---	1:5000	EMD Millipore, Temecula, CA, USA	AB5605
Nitro-Tirosine	Rabbit	---	1:2500	Cell Signaling Technology, Danvers, MA, USA	9691S
Mouse	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3562
Rabbit	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3687
Goat	Rabbit	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A4187

Abbreviations: 4-HNE- 4 Hydroxynonenal; DNP- Dinitrophenol; GLUT1- Glucose transporter 1; GLUT2- Glucose transporter 2; GLUT3- Glucose transporter 3; LDH- Lactate dehydrogenase; MCT4- Monocarboxylate transporter 4; P-mTOR- Phospho-mTOR.

Lactate dehydrogenase (LDH) activity

The commercial Pierce LDH Kit (Thermo Scientific, Rockford, USA) was used to measure LDH activity. The obtained values of activity were calculated using the molar absorptivity of formazan ($\mathcal{E}$ = 19900 $M^{-1}.cm^{-1}$).

Measurement of oxidative damage

The oxidative damage to proteins and lipids was evaluated by Slot-blot. We determined the protein carbonyl, nitro-tyrosine (NT) and 4-hydroxynonenal (4-HNE) group levels as previously described [17]. The membranes were incubated with primary and secondary antibodies (Table 2). ECF detection system was used, the membranes were read in the BioRad FX-Pro-plus and the densities from each band were quantified using Image Lab (BioRad, Hemel Hempstead, UK).

Glutathione content assay

Glutathione content of cells exposed to GLP-1 was performed using the commercial kit to quantify total, oxidized and reduced glutathione contents (Enzo Life Sciences, Lausen, Switzerland), according to the manufactory instructions.

Mitochondrial membrane potential

The cation JC-1 dye (Molecular Probes, Eugene, OR, USA) was used to evaluate mitochondrial membrane potential. Cells were exposed to the chosen concentrations of GLP-1 for 6 hours and mitochondrial membrane potential determined as previously described [18]. Fluorescent intensity was measured using a Cytation^3 Imaging Reader (BioTek Instruments, Winooski, USA). The ratio of the fluorescent intensity of JC-1 aggregates to monomers was used as an indicator of mitochondria membrane potential.

Nuclear Magnetic Resonance (NMR) spectroscopy

The concentration of extracellular metabolites present in the media was determined by ^{1}H-NMR spectroscopy, as previously described [29]. For internal reference we used sodium fumarate (singlet at 6.50 ppm) to quantify the following metabolites (multiplet, ppm): H1-α-glucose (doublet, 5.22), lactate (doublet, 1.33), pyruvate (singlet, 1.35), alanine (doublet, 1.45), acetate (singlet, 1.90). The spectral analysis was done offline using the NUTSpro^{TM} NMR (Acorn, Fremont, USA) to quantify the relative areas of ^{1}H-NMR resonances by a curve-fitting routine.

Statistical analysis

Statistical significance was assessed by one-way ANOVA. All results were performed in triplicate and data are shown as mean ± SEM (n = 6 for each condition). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered significant.

Results

GLP-1 receptor is expressed in human Sertoli cells

GLP-1R was previously identified in the mice testis, only by immunohistochemistry [8]. We investigated the presence of GLP-1R in human Sertoli cells and detected a 300 bp amplicon, corresponding to the presence of GLP-1R mRNA (Figure 1, Panel A). Our results also show that, when exposed to the different concentrations of GLP-1, human Sertoli cells do not alter the transcript levels of GLP-1R (Figure 1, Panel B).

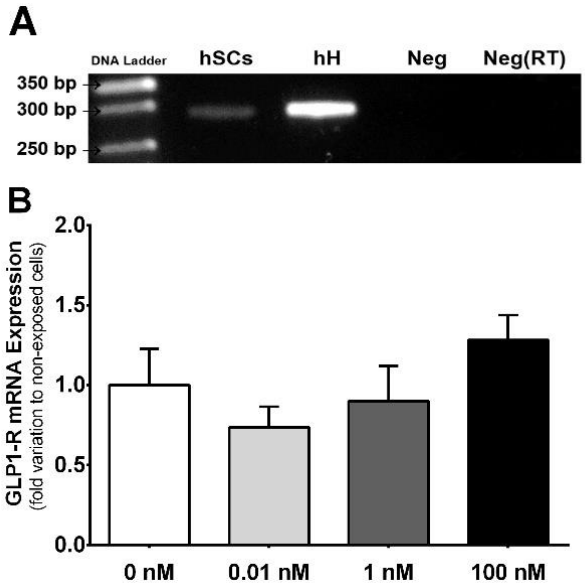

Figure 1. Identification and effects of exposure to glucagon like peptide-1 (GLP-1) in its receptor (GLP-1R) in human Sertoli cells. Panel A - Expression of GLP-1R in cultured human Sertoli cells identified by reverse transcriptase polymerase chain reaction. Panel B - Expression of mRNA transcript levels of GLP-1R in human Sertoli cells exposed to increasing concentrations of GLP-1. Panels B shows pooled data of independent experiments, indicating the mRNA transcript levels of GLP-1R. hSCs: human Sertoli cells; hH: human heart; Neg: Negative control; Neg (RT): Negative without reverse transcriptase. Results are expressed as mean ± SEM (n = 6 for each condition).

Glucose consumption is decreased in human Sertoli cells exposed to GLP-1

Glucose is essential for Sertoli cells metabolism and spermatogenesis. This metabolite enters Sertoli cells mostly through glucose transporters (GLUTs), GLUT1-3 [17, 18]. After treatment with GLP-1, no alterations were detected in the protein levels of those GLUTs in human Sertoli cells (Figure S1, Panel A to C). Nevertheless, when human Sertoli cells were exposed to 0.01 and 1 nM of GLP-1, glucose consumption decreased to 45.35 ± 18.40 and 25.66 ± 15.60 pmol/cell, respectively, while cells exposed to 100 nM of GLP-1 consumed 75.82 ± 28.40 pmol of glucose/cell and those not exposed to GLP-1 consumed 122.30 ± 30.70 pmol of glucose/cell

(Figure 2, Panel A). Sertoli cells also produce acetate [30], which is exported to the intratubular fluid by monocarboxylate transporters (MCTs), as is lactate. Our results show that human Sertoli cells exposed to all GLP-1 concentrations did not alter the protein levels of MCT4 (Figure S1, Panel D). On the other hand, acetate production by human Sertoli cells exposed to 1 nM of GLP-1 increased to 2.49 ± 0.38 pmol/cell when compared to non-exposed cells that produced 1.34 ± 0.31 pmol/cell of acetate (Figure S2, Panel C).

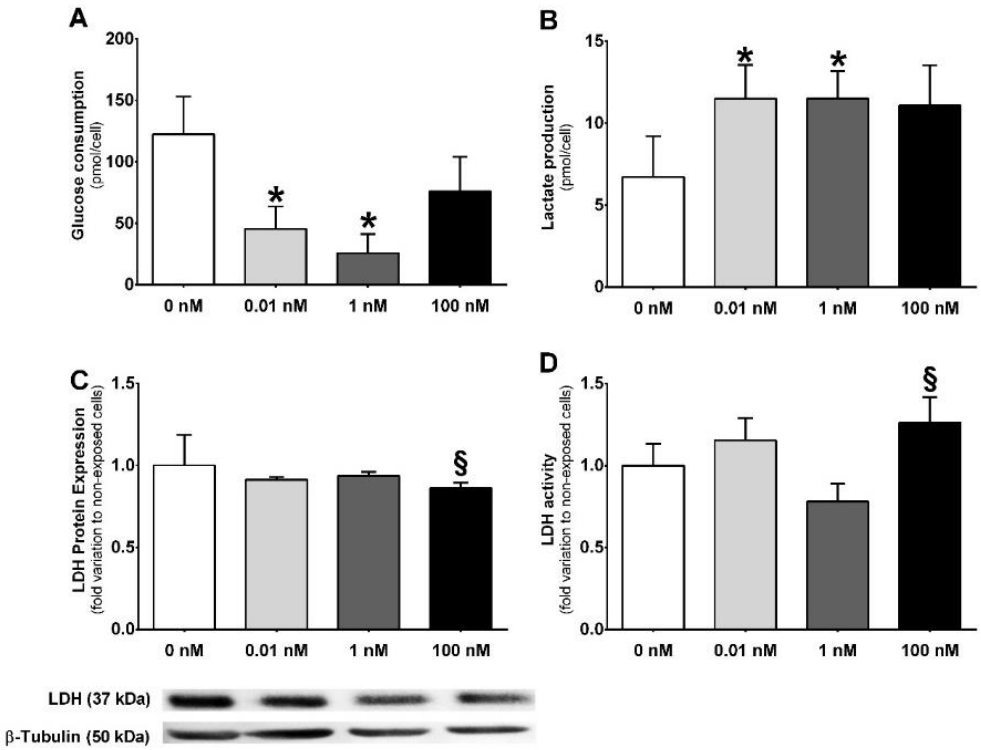

Figure 2. Effects of glucagon like peptide-1 (GLP-1) in glucose metabolism of human Sertoli cells. Glucose consumption (Panel A), lactate production (Panel B), expression of lactate dehydrogenase (LDH) (Panel C) and LDH activity (Panel D) in human Sertoli cells after exposure to increasing concentration of GLP-1 are represented. Panels A and B show pooled data of independent experiments, indicating the glucose consumption and lactate production, respectively. Panels C and D show pooled data of independent experiments, indicating the protein levels and activity of LDH, respectively. Panels C also show representative Western Blot experiments. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($p < 0.05$) are as indicated: * relative to non-exposed cells (no GLP-1); § relative to 1nM.

Exposure to GLP-1 increased lactate production by human Sertoli cells

Once inside the cell, glucose is metabolized to pyruvate. Pyruvate is at a crossroad of several metabolic pathways. Exposure of human Sertoli cells to GLP-1 did not alter the consumption of pyruvate (Figure S2, Panel A). One of the metabolic pathways originated from pyruvate is the

conversion to alanine by alanine aminotransferase. When we evaluated alanine production by human Sertoli cells exposed to GLP-1 no differences were observed (Figure S2, Panel B). In these cells, the majority of pyruvate is converted to lactate by LDH. Our results showed that lactate production by human Sertoli cells exposed to GLP-1 increased to 11.49 ± 2.06, 11.49 ± 1.67 pmol/cell for cells exposed to 0.01, 1 nM, respectively, when compared to non-exposed human Sertoli cells (6.72 ± 2.48 pmol/cell of lactate) (Figure 2, Panel B). We further analysed LDH expression and detected that it slightly decreased in cells exposed to 100 nM of GLP-1 to 0.86 ± 0.03-fold variation to non-exposed cells when compared to cells exposed to 1 nM of GLP-1 (0.94 ± 0.02-fold variation to non-exposed cells (Figure 2, Panel C). However, LDH activity was increased in human Sertoli cells exposed to 100 nM of GLP-1 to 1.26 ± 0.16-fold variation to non-exposed cells and when compared to human Sertoli cells exposed to 1 nM of GLP-1, which, presented an activity of 0.78 ± 0.11-fold variation to non-exposed cells (Figure 2, Panel D).

Human Sertoli cells exposed to 100 nM of GLP-1 presented decreased mitochondrial membrane potential

Our data showed that mitochondrial membrane potential decreased in Sertoli cells exposed to 100 nM (ratio of 0.61 ± 0.02), when compared to non-exposed or those exposed to 0.01 and 1 nM of GLP-1 (ratio of 1 ± 0.06; 1.15 ± 0.07 and 1.11 ± 0.10, respectively) (Figure 3, Panel A). Still, when we analysed the relative quantity of mitochondrial DNA no alteration was observed in human Sertoli cells exposed to GPL-1 when compared to non-exposed cells (Figure 3, Panel B).

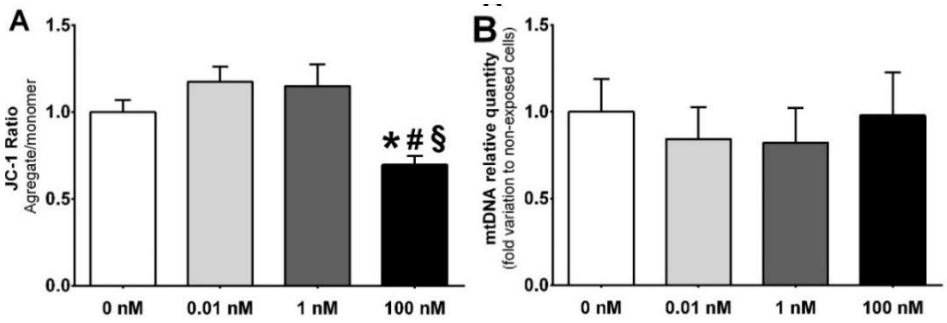

Figure 3. Effects of glucagon like peptide-1 (GLP-1) in mitochondria function of human Sertoli cells. JC-1 ratio (Panel A) and mitochondrial relative copies (Panel B) in human Sertoli cells exposed to increasing concentrations of GLP-1 are represented. Panel A shows JC-1 ratio found in human Sertoli cells cultured with increasing concentrations of GLP-1 or without GLP-1. Panel C shows pooled data of independent experiments indicating mRNA transcript levels as detected by quantitative PCR. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($p < 0.05$) are as indicated: * relative to non-exposed cells (no GLP-1); # relative to 0.01nM; § relative to 1nM.

Carbonyl group levels decreased in human Sertoli cells exposed to 1 and 100 nM when compared to 0.01 nM of GLP-1

The high glycolytic flux, followed by the conversion of pyruvate to lactate, as happens in Sertoli cells, enhances a pro-oxidative environment. The products of protein carbonylation (dinitrophenol (DNP)), nitration (NT) and lipid peroxidation (4-HNE) are biomarkers for oxidative stress. Carbonyl group levels did not alter in any of human Sertoli cells exposed groups to GLP-1 when compared with the no GLP-1 group. However, human Sertoli cells exposed to 1 and 100 nM of GLP-1 presented lower levels of carbonyl groups (0.78 ± 0.07 and 0.86 ± 0.05-fold variation to non-exposed cells (no GLP-1), respectively (Figure 4, Panel A)) when compared with human Sertoli cells exposed to 0.01 nM of GLP-1 (1.10 ± 0.02-fold variation to non-exposed cells (no GLP-1)). Yet, the levels of 4-HNE and NT groups were not altered in cells exposed to GLP-1 when compared with non-exposed cells (Figure 4, Panel B and C, respectively). Additionally, we determined the protein levels of antioxidant enzymes such as catalase and the total/reduced glutathione ratio. Our results show that cells exposed to all GLP-1 concentrations did not presented alterations in the levels of catalase or in the total/reduced glutathione ratio when compared with the levels detected in non-exposed cells (Figure S3, Panel A and B, respectively).

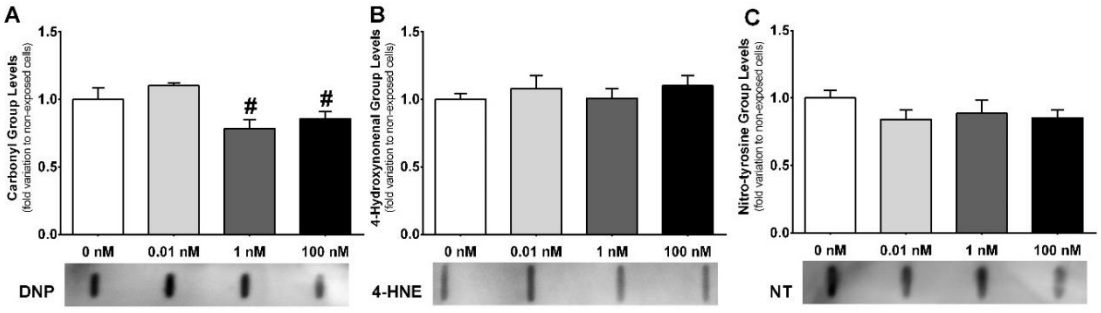

Figure 4. Detection of oxidative damage in human Sertoli cells exposed to increasing concentrations of glucagon like peptide-1 (GLP-1). The levels of carbonyl (Panel A), 4-hydroxynonenal (4-HNE) (Panel B) and nitro-tyrosine (NT) (Panel C) groups after exposure of human Sertoli cells to increasing concentrations of GLP-1 are represented. Panels A to C show pooled data of independent experiments, indicating the expression levels of carbonyl, 4-HNE and NT, respectively. Panels A-C also show representative Slot blot experiments. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($p < 0.05$) are as indicated: # relative to 0.01nM.

Phosphorylated mTOR is increased in human Sertoli cells exposed to 100 nM of GLP-1

mTOR has a crucial role in coordinating cellular homeostasis and energy status [31]. Recent studies demonstrate that mTOR signalling modulates the glycolytic oxidative profile in human Sertoli cells [16]. GLP-1 had no effects regarding mTOR phosphorylation in human Sertoli cells

of any of the exposed groups when compared with expression in human Sertoli cells non-exposed to GLP-1. Still, human Sertoli cells exposed to 100 nM of GLP-1 presented an increase in the protein levels of phosphorylated mTOR (Ser2448), to 1.41 ± 0.28-fold variation to non-exposed cells (no GLP-1) when compared to human Sertoli cells exposed to 0.1 nM of GLP-1 (0.77 ± 0.12-fold variation to non-exposed cells (no GLP-1)) (Figure 5).

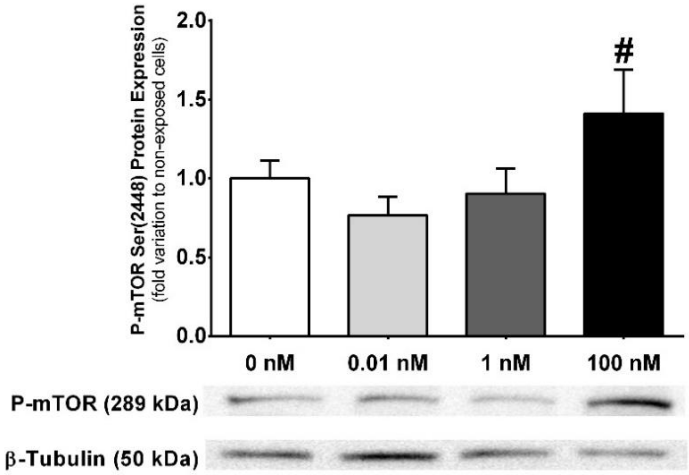

Figure 5. Effect of glucagon like peptide-1 (GLP-1) in mammalian target of rapamycin (mTOR) signaling pathway. The protein expression levels of phosphorylated mTOR (Ser2448) in human Sertoli cells after exposure to increasing concentrations of GLP-1 are represented. Figure shows pooled data of independent experiments, indicating the expression levels of P-mTOR. Figure also show representative Western Blot experiments. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($p < 0.05$) are as indicated: # relative to 0.01 nM.

Discussion

GLP-1 is a peptide hormone with an active role in the regulation of circulating glucose levels [32]. Glucose equilibrium is crucial for energy homeostasis and the proper functioning of physiological functions, including male fertility [33]. Excessive fat accumulation is associated with dysregulation of energy homeostasis signalling systems, and with a concurrent impairment of GLP-1 mediated functions [34]. Several GLP-1 analogues are now used as pharmacological agents to promote weight loss [35]. Recent studies have consistently associated the increase in obesity rates with decreased male fertility [36]. In 2030, it is estimated that over 50% of the world population will be overweight/obese [37], which will aggravate male infertility trends. Alterations in Sertoli cell metabolism were hypothesized to contribute for reduced fertility in obese males [38]. In fact, the Sertoli cells are responsible for the nutritional support of spermatogenesis.

There is evidence that GLP-1 may influence male reproductive function [39], although few studies have been focused on the molecular mechanisms. Since Sertoli cells are major hormonal targets

within the testis [17, 18, 27], we hypothesized that GLP-1 could directly modulate the metabolic functions of these cells with a possible effect on male fertility. For that, we exposed human Sertoli cells to increasing doses of GLP-1 to evaluate: (1) the impact of the postprandial levels of GLP-1; (2) the impact of GLP-1 when present at the levels reached by its analogues (liraglutide) in the plasma of healthy/obese individuals. The maximum plasma concentrations found in overweight and obese subjects treated with GLP-1 analogues is similar to the one observed in healthy individuals [40]. GLP-1 actions are mediated by the GLP-1R. So far GLP-1R has only been identified in mice Sertoli cells by immunohistochemistry of the testicular tissue [8]. Our results allowed us to observe for the first time the expression of GLP-1R in isolated human Sertoli cells. Interestingly, GLP-1 exposure did not change GLP-1R expression in cultured human Sertoli cells, illustrating that the mRNA expression of this receptor in these cells is not rapidly responsive to exposure to increasing GLP-1 concentrations.

We then evaluated the impact of GLP-1 postprandial levels in human Sertoli cells metabolism and bioenergetics. These cells uptake glucose from the interstitial fluid by the action of GLUTs (GLUT1-3). Glucose is then used to produce essential metabolites (mainly lactate) that will serve as energy sources for developing germ cells [17, 18]. Although GLP-1 exposure did not alter GLUTs expression, human Sertoli cells decreased the consumption of glucose after exposure to postprandial levels of this hormone, as compared to non-exposed cells. Concurrent results had been reported in other mammalian cell lines, with GLP-1 exposure decreasing deoxyglucose uptake [41]. Our results suggest that this hormone is capable of eliciting an alteration of glucose uptake, likely due to an alteration on its metabolism. Still, GLP-1 postprandial levels were capable to stimulate lactate production by human Sertoli cells. Previous studies, show that lactate is essential for spermatogenesis [42] since it is used as metabolic fuel by developing germ cells [43] and has an anti-apoptotic effect on those cells [44]. The postprandial levels of GLP-1 increased the production of lactate, while glucose consumption was decreased, as compared to non-exposed cells, illustrating the metabolic commitment of these cells to an efficient production of lactate. No effect was detected on mitochondria functionality in these cells. Neither mitochondrial membrane potential nor DNA content were affected by exposure to these levels of GLP-1. Moreover, the oxidative stress markers (protein carbonyl and nitro-tyrosine groups, and lipid peroxides) were not altered, which correlates with a normal functioning of the mitochondria. Hence, at postprandial levels, GLP-1 seems to be vital for eliciting the production of lactate by human Sertoli cells, which in turn consume lower amounts of glucose. These cells have adaptive mechanisms and show a metabolic plasticity [17, 18, 45, 46] that is very important to sustain the metabolic support of spermatogenesis. They utilize a wide sort of metabolic sources to produce

lactate, namely from triglycerides that accumulate as lipid droplets [47]. Depending on the substrate availability and on stimuli, Sertoli cells can oxidize these cytoplasmic lipidic droplets to support their metabolic requirements [48]. It has been described that GLP-1 may promote lipid droplet remodelling and lipolysis in human adipocytes, when present at concentrations above 10^{-11} M [49]. A similar event may be occurring in human Sertoli cells exposed to the postprandial levels of GLP-1 [21, 22].

Similar results were obtained when the human Sertoli cells were exposed to GLP-1 concentrations that mimic the levels of GLP-1 analogues observed in the plasma after a single administration of liraglutide at the therapeutic dosage recommended for obese individuals [24]. Both glucose consumption and lactate production were maintained, when compared to cells exposed to postprandial levels of GLP-1. The Sertoli cell is responsible for producing lactate favouring the glycolytic flux [50], but it also uses mitochondrial oxidative phosphorylation to sustain its own energetic needs. Human Sertoli cells exposed to 1 nM of GLP-1 presented an increase in acetate production, however this did not lead to an increase in mitochondrial membrane potential, as observed in other cell lines [41]. No changes were observed in the pro-oxidant environment, nitration and lipid peroxidation, nor in the levels of antioxidant defences, but we found less oxidative damage in proteins of cells exposed to this concentration of GLP-1. This protective effect mediated by GLP-1 may be essential to counteract the pro-oxidant environment promoted by an increased metabolic activity.

Contrasting results were obtained with cells exposed to GLP-1 concentrations that mimic the levels of GLP-1 analogues observed in the plasma after a prolonged administration of liraglutide at the therapeutic dosage recommended for obese individuals [23, 25]. In this case, human Sertoli cells appear to consume higher amounts of glucose, producing the same amounts of lactate, as compared to cells exposed to postprandial GLP-1 levels. This increased glucose consumption was associated with LDH activity stimulation, which suggest an adaptation to sustain lactate production. In fact, human Sertoli cells exposed to 100 nM of GLP-1, presented a decrease in mitochondrial membrane potential. As said, Sertoli cells have a distinct metabolic behaviour when compared to most somatic cells, with remarkable metabolic resemblances with cancer cells [50]. Thus, decreased mitochondrial membrane potential may not be a sign of compromised function, but rather a shift to sustain the metabolic biosynthetic requirements of developing germ cells and redirecting the metabolism of the cell for oxidative phosphorylation. Indeed, when we accessed oxidative damage, which is often associated with mitochondria malfunction, the exposure to 100 nM GPL-1 did not promote protein nitration nor lipid peroxidation, but these cells rather showed less oxidative damage in proteins without any changes in the levels of antioxidant defences.

Again, GLP-1 appears to exert a protective effect that is essential to counteract testicular pro-oxidant environment. Although these results suggest that GLP-1 has an antioxidant effect the exact mechanism by which GLP-1 decreases oxidative stress in human Sertoli cells remains to be elucidated. Our studies further suggest that these alterations in glucose metabolism and mitochondrial function in human Sertoli cells exposed to 100 nM GPL-1 are associated with stimulation of mTOR Complex 1 (mTORC1) pathway, as mTOR phosphorylation at Ser2448 is increased. Although the significance of mTORC1 pathway for GLP-1 mediated effects is unclear, the involvement of this signalling pathway has been described in several cellular systems [51].

In conclusion, GLP-1 was able to modulate glucose metabolism and bioenergetics, promoting the production of lactate by human Sertoli cells. Moreover, exposure to the highest concentration of GLP-1 decreased oxidative damage in these cells. Also, the absence of toxic effects of GLP-1 at this concentration in human Sertoli cells, allied to a decrease in oxidative damage, adds a possible positive impact on male fertility. Still, further experiments are needed to clarify the effects of GLP-1 in male reproductive health and to determine if the effects observed *in vitro* translate to *in vivo*. Taking in consideration the decline of fertility rates parallel to the increasing prevalence of obesity, it is crucial to understand how GLP-1 affects male fertility. The use of GLP-1 analogues for obesity treatment could also be valuable to counteract the negative impact of adiposity related metabolic dysregulation in male reproductive function and arise as an additional target for medical intervention.

Supplemental Data

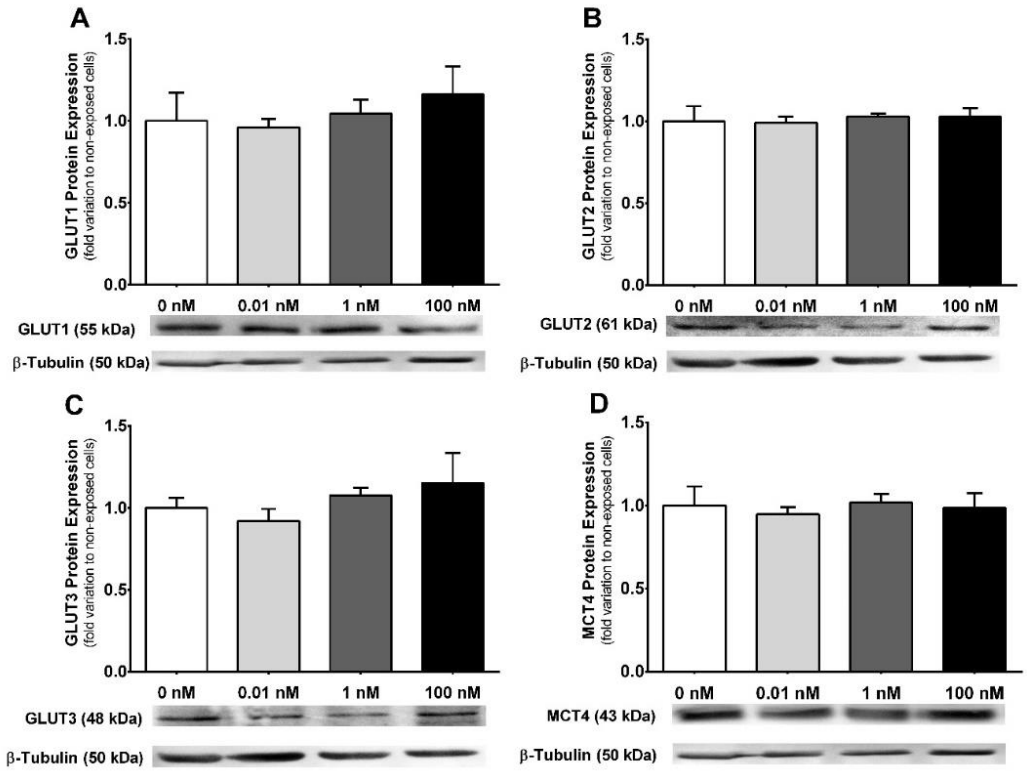

Figure S1. Effect of glucagon like peptide-1 (GLP-1) in glucose metabolism related transporters in human Sertoli cells. Expression of glucose transporter 1 (GLUT1) (Panel A) and glucose transporter 2 (GLUT2) (Panel B) and glucose transporter 3 (GLUT3) (Panel C) and monocarboxylate acid transporter 4 (MCT4) (Panel D) by human Sertoli cells after exposure to GLP-1 are represented. Panels A to D show pooled data of independent experiments, indicating the protein levels of GLUT1, GLUT2, GLUT3 and MCT4, respectively. Panels A-D also show representative Western Blot experiments. Results are expressed as mean ± SEM (n =6 for each condition).

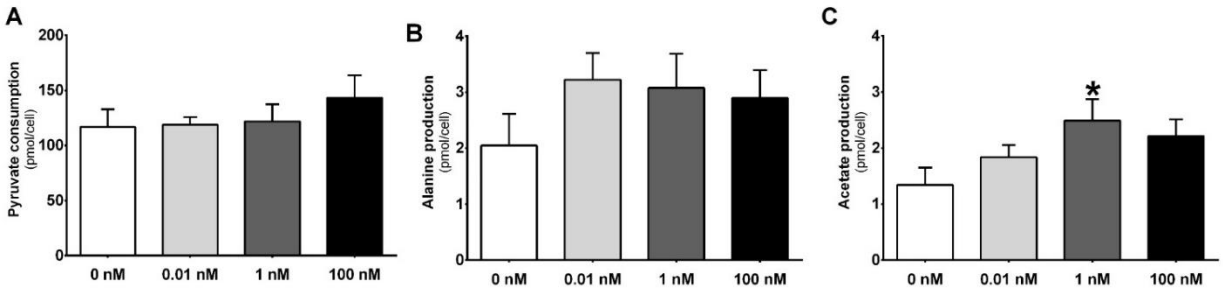

Figure S2. Effect of glucagon like peptide-1 (GLP-1) in pyruvate consumption and alanine and acetate production in human Sertoli cells. Lactate consumption (Panel A), alanine (Panel B) and acetate (Panel C) production by human Sertoli cells after exposure to GLP-1 is represented. Panel A to C show pooled data of independent experiments, indicating the consumption of lactate, and production of alanine and acetate, respectively. Results are expressed as mean ± SEM (n =6 for each condition). Significantly different results ($p < 0.05$) are as indicated: * relative to non-exposed cells.

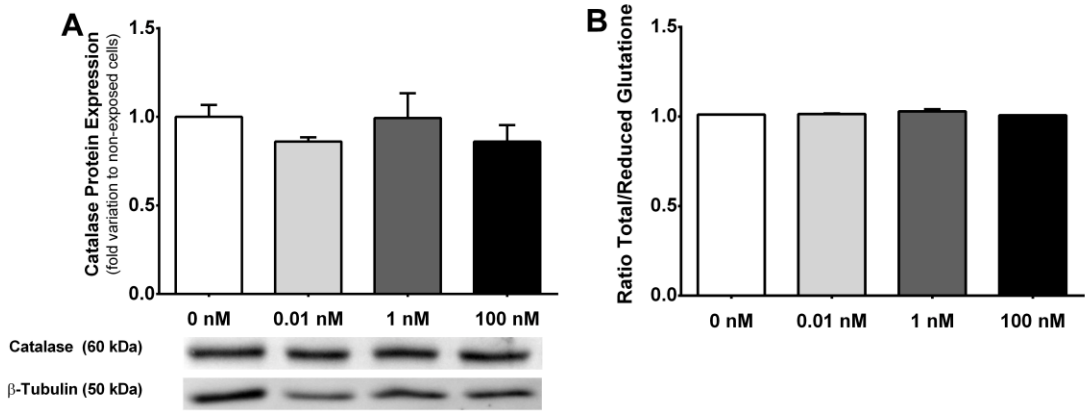

Figure S3. Effect of glucagon like peptide-1 (GLP-1) in protein levels of catalase and glutathione content in human Sertoli cells. Protein levels of catalase (Panel A) and ratio of total/reduced glutathione (Panel B) in human Sertoli cells after exposure to GLP-1 is represented. Panel A and B show pooled data of independent experiments, indicating the protein levels of catalase and glutathione total/reduced ratio, respectively. Results are expressed as mean ± SEM (n =6 for each condition).

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Chapter 8

Caloric restriction alters the hormonal profile and testicular metabolome resulting in alterations of sperm morphology

This chapter was adapted from the submited work:

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Caloric restriction alters the hormonal profile and testicular metabolome resulting in alterations of sperm morphology

Abstract

Energy homeostasis is crucial for all physiological processes. During energy deprivation conditions negative effects may arise, among which in reproductive function. We propose to study whether caloric restriction (CR) changes testicular metabolic profile and ultimately sperm quality. Male Wistar rats (n=12) were randomized into a CR group fed with 30% less calories than weight matched *ad libitum* fed animals (control group). Circulating hormonal profile, testicular glucagon like peptide-1 (GLP-1), ghrelin and leptin receptors expression and sperm parameters were analyzed. Metabolites and glycolysis related enzymes in testicular tissue were determined by NMR and Western blot, respectively. Oxidative stress markers were analyzed in testicular tissue and spermatozoa. Testicular mitochondrial biogenesis and expression of mitochondrial complexes were determined. CR induced changes in body weight along with altered GLP-1, ghrelin and leptin circulating levels. In testis, CR led to changes in receptors expression that followed those of the hormone levels; modified testicular metabolome particularly amino acids content; decreased oxidative stress-induced damage in testis and spermatozoa though increasing sperm head defects. CR induced changes in body weight, altering circulating hormonal profile, testicular metabolome and increasing sperm head defects. Ultimately, our data highlights that conditions of CR may compromise male fertility.

Introduction

Energy availability is a critical factor for health and survival of all living species and depends upon food disposal. Indeed, the availability of nutrients is of extreme importance for a correct functioning of all physiological processes and most particularly to ensure normal reproductive function [1]. In conditions of energy insufficiency, such as those induced by caloric restriction (CR), several deleterious health effects may arise, being particularly notorious and well described in female reproductive function [2]. Furthermore, the metabolic input changes the endocrine signaling in brain, including those from hormones known to have an important role in the testicular function, such as leptin, ghrelin, glucagon like peptide-1 (GLP-1), testosterone, and insulin [3-8].

Thus, CR can have a potential negative impact on spermatogenesis and sperm production, although the molecular mechanism leading to these effects remains largely unknown.

Current literature suggests that CR in young adult male rhesus macaques has a low influence on the expression of genes with an important role in testis, such as uncoupling protein 2, cyclooxygenase 2 and casein kinase 1 epsilon [9]. In addition, the authors report no effects on testosterone levels [9, 10] and semen quality [10]. Contrastingly, Cameron and Nosbisch demonstrated that brief periods of undernutrition can suppress the reproductive axis in male rhesus monkeys [11]. The effects of nutrition have also been studied in adult male rams, and it was shown that nutrition affects the spermatogenic efficiency [12]. Thus, the effect of CR in male fertility remains controversial. Herein we aim to study if CR alters testicular metabolome and sperm quality. For that, we subjected juvenile male Wistar rats to a 28 days CR and performed a NMR-based metabolomics analysis together with molecular biology studies to determine the testicular metabolome, hormonal receptors expression and markers of oxidative damage.

Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, USA) except stated otherwise.

Animals

Twelve 6-week-old male Wistar rats (Charles River Laboratories, Barcelona, Spain) were randomly selected from our colony. The rats were housed in our accredited animal colony and maintained with food and water *ad libitum* in a constant room temperature (20 ± 2°C) on a 12-h cycle of artificial lighting. All animal experiments were performed according the ARRIVE guidelines and was licensed by the Portuguese Veterinarian and Food Department (0421/000/000/2016).

Experimental design

Body weight matched rats were randomly divided into two groups (six *per* group): to be subjected to CR or used as controls. Rats in the control were fed *ad libitum* with a standard chow diet (4RF21 certificate, Mucedola, Italy) while the rats subjected to CR received 30% less chow diet than the former for 28 days. Food consumption and the animal's weight were daily monitored. After the experimental period, animals were euthanized by carbon monoxide and killed by exsanguination after cardiac puncture. Blood was collected into chilled EDTA tubes, centrifuged and plasma was

stored at -20ºC until assayed. Testes were removed and processed for histochemistry, protein, DNA, RNA and metabolites extraction and stored at -80°C until further use.

Hormonal measurements

Plasma leptin (cat# EZRL-83K, detection range: 0.2-30 ng/mL), ghrelin (cat# EZRGRT-91K, detection range: 0.04-10 ng/mL) and GLP-1 levels (cat# EZGLP1T-36K, detection range: 4.1-1000 pM) were measured using commercial kits (Millipore, Billerica, MA, USA) following the suppliers' instructions.

Epididymal sperm parameters

Epididymis were isolated and placed in pre-warmed (37°C) Hank's Balanced Salt solution (pH 7.4), crushed with a stiletto blade and incubated for 5 min (37°C). Eosin-nigrosin staining was used to assess sperm viability examining epididymal sperm smears. Dead spermatozoa stained pink, due to compromised membrane integrity, while viable spermatozoa appear in white. A total of 1000 spermatozoa were counted under a light microscope. Epididymal sperm concentration was determined using a Neubauer counting chamber. Diff-Quick (Baxter Dale Diagnostics AG, Dubinger, Switzerland) stained smears were used to assess sperm morphology according to manufacturers' instructions. Sperm cell with hook-shaped head and no defects of head, neck or tail were classified as normal. Otherwise, spermatozoa were considered abnormal. Remaining sperm from the epididymis was washed with PBS and stored at -80°C.

Histochemistry evaluation of seminiferous tubule diameter

Five-micrometre sections of rat testis from the experimental groups were fixed in 4% paraformaldehyde. For each animal, two slides were stained with haematoxylin-eosin and analysed. The mean tubule diameter was derived in 25 round transverse section of seminiferous tubules *per* animal by taking the average of two diameters, D_1 and D_2 at right angles where $D_1/D_2 \geq 0.85$ [13].

Protein extraction and quantification

M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, USA) was used to extract total protein. About 50 mg of testicular tissue was weighed and homogenized in the M-PER mammalian protein extraction reagent. For sperm protein extraction the remaining pellet of epididymal sperm was homogenised in extraction reagent. In both cases, manufacturers'

instructions were followed. Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA) according to the manufacturers' instructions.

Western blot

Western blot was performed according to our laboratory protocol [14], using 50 µg of extracted protein. PVDF membranes were incubated with primary and secondary antibodies, at 4ºC overnight and at room temperature for 90 minutes, respectively (Table 1). The membranes were incubated with Restore PLUS Western Blot Stripping Buffer (Thermo Scientific, Rockford, USA) when appropriated to reproving the membrane. For protein loading control mouse β-actin or Ponceau Staining (NZYTech, Lisboa, Portugal) was used. For detection, ECL (NZYTech, Lisboa, Portugal) was used and membranes were exposed in BioRad FX-Pro-plus (BioRad, Hemel Hempstead, UK). Image lab software (BioRad, Hemel Hempstead, UK) was used to quantify the bands` density.

Lactate dehydrogenase (LDH) activity

LDH activity was measured using a commercial Pierce LDH Kit (Thermo Scientific, Rockford, USA) and performed by using 5 mg of extracted protein according to the manufacturers' instructions. The values of activity were calculated using the molar absorptivity of formazan (Ɛ=19900 $M^{-1}.cm^{-1}$).

Measurement of damages caused by oxidative stress

Evaluation of oxidative damages to proteins and lipids was performed by Slot-blot to determine protein carbonyl, nitro-tyrosine (NT) and 4-hydroxynonenal (4-HNE) levels, according to the standard protocol used in our laboratory [6]. PVDF membranes were incubated overnight at 4°C with primary antibodies (4-HNE, NT and dinitrophenol (DNP)) and then 90 minutes at room temperature with secondary antibodies (Table 1). The detection mode, acquisition and quantification were performed as described for Western blot.

RNA and DNA extraction

To extract total RNA and DNA from rat testis and spermatozoa, the E.Z.N.A.® Total RNA commercial Kit and E.Z.N.A.® Tissue DNA Commercial Kit (both from Omega Bio-Tek, Norcross, USA) were used, according to the manufacturers' instructions.

Table.1. List of antibodies used in this study

Antibody	Source	Molecular Weight (kDa)	Dilution	Vendor	Catalog #
GLUT1	Rabbit	55	1:500	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7903
GLUT2	Rabbit	61	1:5000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-9117
GLUT3	Goat	48	1:500	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7582
MCT4	Rabbit	43	1:1000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-50329
LDH	Rabbit	37	1:10000	Abcam, Cambridge, MA, USA	ab52488
S6RP	Rabbit	32	1:500	Cell Signalling Technology, Danvers, MA, USA	2217S
P-mTOR	Rabbit	289	1:500	Cell Signalling Technology, Danvers, MA, USA	2971S
Total OXPHOS	Mouse	20, 30, 40, 48, 50	1:1000	Abcam, Cambridge, United Kingdom	ab110413
β-actin	Mouse	32	1:5000	Thermo Fisher Scientific, Rockford, USA	MA5-15739
DNP	Rabbit	---	1:5000	Sigma-Aldrich, Roedermark, Germany	D9656
4-HNE	Goat	---	1:5000	EMD Millipore, Temecula, CA, USA	AB5605
Nitro-Tyrosine	Rabbit	---	1:2500	Cell Signalling Technology, Danvers, MA, USA	9691S
Mouse	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3562
Rabbit	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3687
Goat	Rabbit	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A4187

Abbreviations: 4-HNE: 4 Hydroxynonenal; DNP: Dinitrophenol; GLUT1: Glucose transporter 1; GLUT2: Glucose transporter 2; GLUT3: Glucose transporter 3; LDH: Lactate dehydrogenase; MCT4: Monocarboxylate transporter 4; P-mTOR: Phospho-mTOR; S6RP: S6 Ribosomal Protein

Polymerase Chain Reaction

cDNA was obtained from extracted total RNA by our laboratory protocol [15] using NZY M-MuLV Reverse Transcriptase (NZYTech, Lisboa, Portugal). Specific cDNA fragments were amplified using designed exon-exon spanning primer set (Table 2), with optimal annealing temperatures. Sirtuin 1 (*Sirt1*), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α*), nuclear respiratory factor 1 (*NRF1*), glucagon like peptide-1 receptor (*GLP-1R*), leptin receptor (*OB-R*) and growth hormone secretagogue receptor (*GHS-R*) mRNA levels were evaluated in rat testis, and β-2-microglobulin (*β2M*) transcript levels were used to normalize expression. Target

genes, sequences and annealing temperatures are presented in Table 2. The efficiency of the amplification and qPCR experiments were carried in a CFX96 thermocycler (Biorad, Hercules, USA), using our laboratory protocol [16]. LDH. Fold variation of the expression of target genes was calculated using the mathematical model suggested by Pfaffl in the formula: $2^{-\Delta Ct}$, where ΔCt is the deviation of the control–sample of reference of transcript gene [17].

Table 2. List of primers used in this study

Gene	Primer sequence (5′-3′)	AT(°C)	Amplicon size (bp)
GHS-R	Sense: AGGAAGCTATGGCGGAGACG Anti-Sense: TTGAACACTGCCACCCGGTA	59	296
Ob-R	Sense: GCCATCAATTCCATCGGTGC Anti-Sense: GTCCAGGAAAGGATGACGCA	56	131
GLP-1R	Sense: CCCCCAGGTTCCTTTGTGAA Anti-Sense: TTGCTTGGACTCTTCGCACT	62	168
Sirt1	Sense: ACACACAAAATCCAGCAACTC Anti-Sense: GATGCTGTTGCAAAGGAACCA	60	253
NRF1	Sense: TACTCCACAGGTCGGGGAAA Anti-Sense: CATACAGAAGGTCCTCCCGC	62	199
PPARGC-1α	Sense: TGGAGTGACATAGAGTGTGCTG Anti-Sense: CTGATCCTGTGGGTGTGGTT	56	491
β2M	Sense: CCGTGATCTTTCTGGTGCTTGTC Anti-sense: CTATCTGAGGTGGGTGGAACTGAG	60	150
ND1	Sense: GAGCCCTACGAGCCGTTGCC Anti-Sense: GCGAATGGTCCTGCGGCGTA	60	121
$\beta 2M_{nc}$	Sense: GAGGCTATCCAGCGTGAGTC Anti-sense: GACGCTTATCGACGCCCTAA	60	306

Abbreviations: β2M: Beta-2-Microglobulin; $\beta 2M_{nc}$: nuclear encoded beta-2-microglobulin; GHS-R: Growth hormone secretagogue receptor; GLP-1R: Glucagon like peptide-1 receptor; ND1: NADH dehydrogenase subunit 1, NRF1: Nuclear Respiratory factor 1, Ob-R: Obesity receptor (or Leptin receptor), PPARGC-1α Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, Sirt1: Sirtuin1.

Determination of mtDNA Copy Number

To study mtDNA copy number we used a qPCR analysis in a CFX 96 thermocycler (Biorad, Hercules, USA), as described [8]. Annealing temperatures and primer sequences are presented in Table 2. To quantify the relative mtDNA copy number, we used the model proposed by Pfaffl ($2^{-\Delta Ct}$) [17] were C_t value differences between NADH-ubiquinone oxidoreductase chain 1 (*ND1)* and nuclear encoded beta-2-microglobulin (*$\beta 2M_{nc}$*) were used.

Nuclear Magnetic Resonance spectroscopy

Testicular tissue extracts were prepared using a combined extraction of polar and nonpolar metabolites as previously described [18]. For NMR analysis, extracts were dissolved in D_2O phosphate buffer (0.2 M, pH=7). ^{1}H-NMR spectra were recorded using a Varian Inova 600 MHz (14.1 T) spectrometer equipped with a 3 mm QXI probe with a z-gradient. ^{1}H-1D noesy experiments with water pre-saturation were acquired (7.2 kHz spectral width, 0.1 s mixing time, 4, 4 s relaxation delay with 3 s of water pre-saturation, 90º pulse angle, 3 s acquisition time and 128 scans at 298 K). Pulse durations and water saturation frequencies were optimized for each sample. 2D homonuclear total correlation spectroscopy (TOCSY) spectra were acquired to help signal assignment (sweep width of 5.4 kHz in both dimensions, 400 and 1024 points in t1 and t2 dimensions). Spectra were processed as previously reported [19]. Peaks were assigned by comparing recorded 1D and 2D spectra with reference spectra and public databases such as HMDB [20]. Metabolites were identified according to Metabolomics standards initiative guidelines for metabolite identification and the levels of identification are referenced in Supplementary information Table S1 [21]. Processed 1D noesy spectra were bucketed using one-point bucket (0.6-9.0 ppm, with signal-free, water and fumarate regions excluded). Data matrix was built in Amix Viewer (version 3.9.15, BrukerBiospin, Rheinstetten). Icoshift algorithm [22] was used to align bucketed spectra and total area integral normalization was applied to account for the variations in the overall sample concentrations. Multivariate statistical analysis was applied on unit variance scaled matrix (SIMCA 14, Umetrics, Sweden). PCA was used to provide information on global data structure, and PLS-DA was used to assess class separation and to identify the main metabolites that contribute to the class discrimination. A 7-fold internal cross-validation and permutation test (n=100) of the PLS-DA model were used to provide the qualitative measure of predictive power (Q^2) and to assess the degree of fit to the data (R^2). The corresponding PLS-DA loadings were obtained by multiplying the loading weight factors (w) by the standard deviation of the respective variable and were colour-coded according to variable importance in the projection (VIP). Selected signals of chosen metabolites (VIP>1) were integrated in normalized ^{1}H-NMR spectra for quantitative assessment of metabolite variations between groups.

Statistical analysis

Statistical significance among the experimental groups was assessed by t-test. All results were performed in triplicate and the data are shown as mean ± SEM (n=6 for each condition). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, USA). $p<0.05$ was considered significant.

Results

Caloric restriction decreased body weight and altered the hormonal profile, decreasing sperm quality

As expected, CR led to rats with decreased body weight by 14% (298.0 ± 2.6 g) when compared with the body weight of rats fed *ad libitum* (347.1 ± 6.5 g) (Table 3). After 28 days of CR, blood glucose levels were not different when compared with control rats (Table 3). Nevertheless, CR induced significant changes in hormonal profile, particularly of gut hormones as previously described [23, 24]. Indeed, CR doubled GLP-1 blood levels (44.5 ± 3.5 pM), when compared with levels detected in rats fed *ad libitum* (24.2 ± 3.9 pM) (Table 3). Blood ghrelin levels were also higher in rats subjected to a CR (1.0 ± 0.2 ng/mL), when compared with rats fed *ad libitum* (0.4 ± 0.1 ng/mL) (Table 3). The contrary effect was observed for leptin levels that were significantly lower in CR rats (1.8 ± 0.4 ng/mL) as compared to levels detected in rats fed ad libitum (6.9 ± 0.7 ng/mL) (Table 3).

Table 3: Physiological and sperm parameters measured

Parameter	**Control**	**Caloric Restriction**
Body Weight (g ± SEM)	347.1 ± 6.5	298.0 ± 2.6*
Total GLP-1 (pM ± SEM)	24.2 ± 3.9	44.5 ± 3.5*
Leptin (ng/mL ± SEM)	6.9 ± 0.7	1.8 ± 0.4*
Ghrelin (ng/mL ± SEM)	0.4 ± 0.1	1.0 ± 0.2*
Glucose (mg/dL ± SEM)	89.6 ± 2.2	88.0 ± 3.2
Testis Weight (g ± SEM)	3.6 ± 0.1	3.2 ± 0.2
Seminiferous Tubule Diameter (μm ± SEM)	289.8 ± 2.7	283.9 ± 4.7
Sperm Parameters	**Control**	**Caloric Restriction**
Concentration (x10^6 sperm/mL ± SEM)	38.1 ± 4.6	31.9 ± 4.7
Viability (% viable ± SEM)	42.4 ± 5.8	36.1 ± 2.2
Morphology (%normal ± SEM)	61.0 ± 3.1	59.6 ± 1.7
Neck Defects (% sperm ± SEM)	19.6 ± 3.2	19.1 ± 3.3
Tail Defects (% sperm ± SEM)	13.7 ± 1.1	12.0 ± 2.2
Head Defects (% sperm ± SEM)	3.1 ± 1.0	8.7 ± 1.7*

Significantly different results ($p<0.05$) are as indicated: * relative to control group. Abbreviations: GLP-1: Glucagon like peptide-1. Results are expressed as mean±SEM (n=6 for each condition).

Body weight and hormonal profiles variations are able to modify testicular architecture and epididymal sperm quality [1]. Still, the seminiferous tubules architecture of rats subjected to CR for 28 days did not change, when compared to those of rats fed *ad libitum* (Table 3). Nevertheless, when sperm quality was evaluated, the percentage of spermatozoa with head defects was higher in rats subjected to CR (8.7 ± 1.7%) when compared with rats fed *ad libitum* (3.1 ± 1.0%). No other alterations on sperm quality parameters were observed (Table 3). Although CR had no changes in testicular histological architecture, sperm quality was affected in one of the most relevant parameters, the head morphology, suggesting that molecular changes, mediated by weight loss and the shift on hormonal profile, mediate the deleterious effects detected in sperm. Thus, we analyzed the expression of hormonal receptors in testis of control and rats subjected to CR.

Leptin and ghrelin receptors expression in the testis is concurrent with the levels of these hormones after caloric restriction

Since CR induced strong hormonal changes, we further studied if expression of receptors for those hormones in testis responded to the hormonal changes detected. Hormonal receptors for leptin [25], ghrelin [26] and GLP-1 [27] were previously identified in the testis of rodents. Our results show that 28 days CR did not induce any alteration in the testicular expression of GLP-1 receptor (Figure 1, Panel A). Nevertheless, the expression of leptin and ghrelin receptors in the testis responded to the hormonal levels detected in the blood of rats subjected to CR. Leptin receptor, also known as obesity receptor, increased in testicular tissue of rats subjected to CR to 4.8 ± 1.8-fold variation when compared with the expression detected in the testis of rats fed *ad libitum* (Figure 1, Panel B). Contrastingly, the ghrelin receptor expression was decreased (0.2 ± 0.1-fold variation) in the testicular tissue of rats subjected to CR when compared with the expression detected in the testis of rats fed *ad libitum* (Figure 1, Panel C). These results illustrate that CR alters the hormonal profile, which is reflected in the expression levels of the respective receptors in the testis. We have previously shown that leptin [6], ghrelin [7] and GLP-1 [8] act as energy sensors for the nutritional support of spermatogenesis. Therefore, we analyzed testicular metabolome to unveil metabolic changes in testis caused by CR and mediated by hormonal changes.

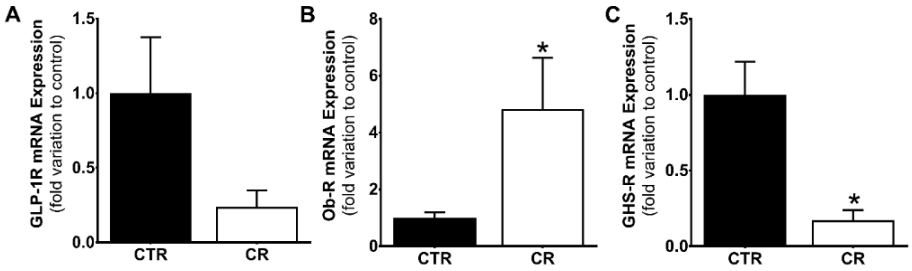

Figure 1. Effect of caloric restriction (CR) during 28 days in mRNA levels of hormonal receptors in testicular tissue. Panel A–Expression of GLP-1R (Glucagon Like Pepetide-1 receptor); Panel B–Expression of Ob-R (Leptin Receptor); Panel C–Expression of GHS-R (Ghrelin Receptor) in testicular tissue of rats fed with CR when compared with rats fed *ad libitum* (CTR). Panels A-C shows pooled data of independent experiments, indicating the mRNA transcript levels of GLP-1R, Ob-R and GHS-R detected by quantitative PCR. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($p<0.05$) are as indicated: * relative to control.

Caloric restriction alters testicular metabolome

The altered hormonal profile induced by CR suggested an overall metabolic dysfunction. Indeed, we have shown that the altered hormones can change the nutritional support of spermatogenesis. Thus, we evaluated the testicular metabolome of rats from both experimental groups by using a ^{1}H-NMR metabolomics-based analysis. PCA analysis was used to examine internal data structure and search for possible outliers and clustering trends (Figure 2, Panel A). Clear separation was observed between the two groups in PLS-DA model as well, and analysis of PLS-DA loadings was used to identify metabolites preferentially expressed in the testis of rats fed *ad libitum* or subjected to a 28 days CR (Figure 2, Panel B). CR induced a distinct metabolic change (Figure 2, Panels A and B) that was not only caused by a general decrease in amino acid content but also some other relevant metabolites (Figure 2, Panel C). The normalized integrated area of alanine (ala), glutamate (glu), proline (pro) and ethanolamine showed a clear decrease in the testis of rats subjected to CR (Figure 3, Panel A-C and Panel E). On the contrary, phosphocholine and hypoxanthine levels were found to be increased in the testis of rats subjected to CR (Figure 3, Panels D and F). Although glycolysis appeared not to be affected, we further analyzed this pathway since it is crucial for testicular metabolism. We detected the levels of enzymes and transporters involved in glycolysis. However, testicular levels of glucose transporter 1 (GLUT1), GLUT2 and GLUT3 (Figure S2, Panel A-C) were not affected, as well as monocarboxylic acid transporter 4 (MCT4) (Figure S2, Panel F). It is known that LDH has also a relevant role in spermatogenesis, since developing germ cells depend upon the lactate synthesized from glucose by Sertoli cells [28, 29]. However, CR has no effect in LDH expression (Figure S2, Panel D) nor in LDH activity in testis (Figure S2, Panel E), which is concomitant with the metabolomics results suggesting that lactate production is not affected by CR.

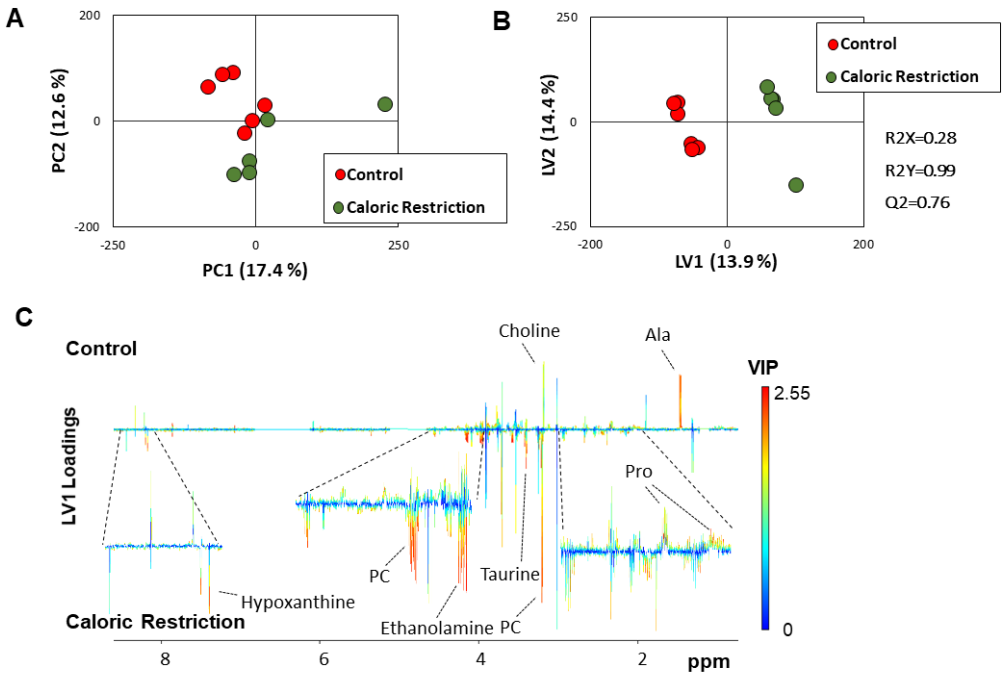

Figure 2. Scores scatter plots obtained by PCA (Panel A) and PLS-DA (Panel B) analysis of ^{1}H-NMR spectra of polar testicular extracts of rats subjected to caloric restriction (CR) when compared with rats fed *ad libitum* (CTR). LV1 loadings extracted from PLS-DA are presented in Panel C. Loadings are colored according to variable importance to the projection (VIP). Abbreviations: PC, phosphocholine; three letter code for amino.

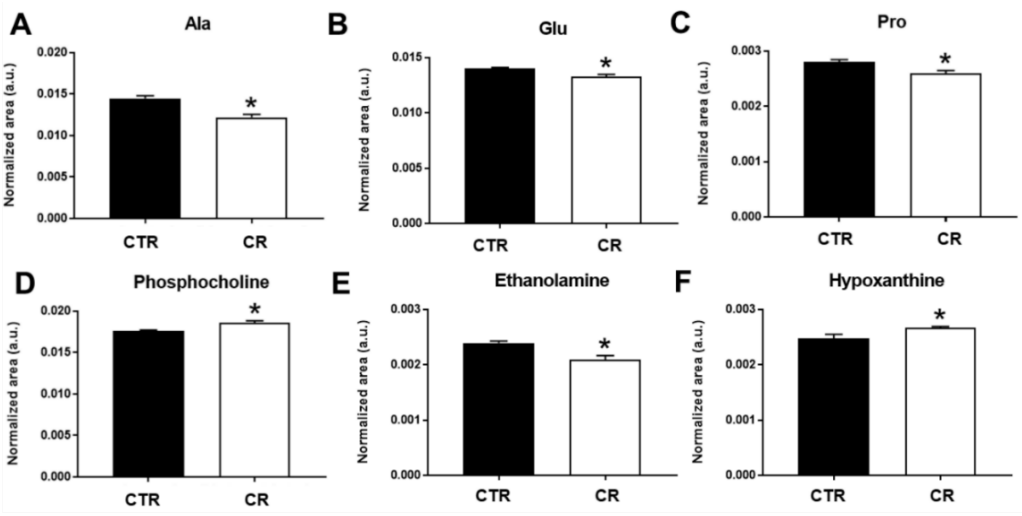

Figure 3. Effect of caloric restriction (CR) during 28 days in testicular tissue content in alanine (Ala), glutamate (Glu), prolanine (Pro), phosphocholine, ethanolamine and hypoxanthine compared with rats fed ad libitum (CTR). Statistical significance was evaluated by the t-test. Testicular content in alanine (Panel A), glutamate (Panel B), prolanine (Panel C), phosphocholine (Panel D), ethanolamine (Panel E) and hypoxanthine (Panel F) of rats fed with CR when compared with CTR rats is shown after normalization by total area integral. Panels A and F show pooled data of independent experiments. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($p<0.05$) are as indicated: * relative to control.

NRF1 expression was upregulated in the testis of rats subjected to caloric restriction

The observed alterations in testicular metabolome caused by CR led us to hypothesize that mitochondria, which respond to the hormonal profile [7, 8], could be mediating the effects. Thus, we analyzed genes related with mitochondrial biogenesis, such as *PGC-1α*, *NRF1* and *Sirt1*. In testicular tissue of rats subjected to CR, *NRF1* expression was found to be upregulated (1.5 ± 0.1-fold variation to control), when compared with the expression detected in the testis of rats fed *ad libitum* (Figure 4, Panel B). Nevertheless, no differences were observed in the expression of *PGC-1α* and *Sirt1* in the testis of both, rats fed *ad libitum* or subjected to CR (Figure 4, Panel A and C). Since alterations in *NRF1* could be reflected in the relative mitochondrial copy number, we quantified mitochondrial content in both testicular tissue (Figure 4, Panel D) and spermatozoa (Figure 4, Panel E), but no differences were observed between the experimental groups. We further determined the protein expression of mitochondrial complexes in testis, which showed no differences either (Figure S3, Panels A-E).

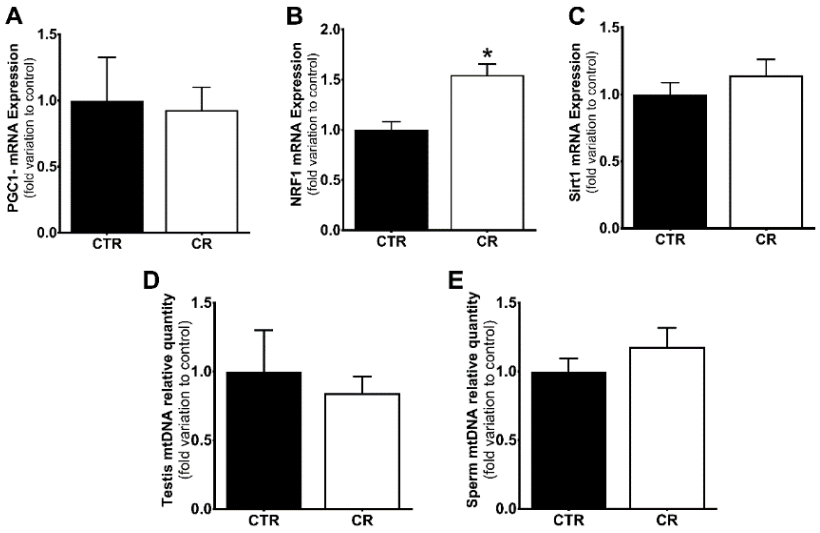

Figure 4. Effect of caloric restriction (CR) during 28 days in testicular tissue in mitochondria biogenesis related genes (Panel A-C) and relative mitochondrial copy number (Panel D) and in spermatozoa relative mitochondrial copy number (Panel E) compared with rats fed *ad libitum* (CTR). Panels A-E shows pooled data of independent experiments, indicating the mRNA transcript levels of PGC1-α, NRF1, Sirt1, GHS-R and mitochondrial copies in testicular tissue and spermatozoa detected by quantitative PCR. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($p<0.05$) are as indicated: * relative to control.

Oxidative damage is decreased in testis and spermatozoa from rats subjected to caloric restriction

Since alterations in the metabolic profile are often associated with oxidative damage, we analyzed markers for oxidative damage in testis and spermatozoa. Thus, some specific biomarkers, such as carbonyl, NT and 4-HNE substituted proteins were measured in testicular tissue of rats fed *ad*

libitum or subjected to CR. Our results show that CR induced a decrease in the testicular levels of carbonyl groups (0.69 ± 0.07-fold variation) (Figure 5, Panel A) as well as NT levels in testicular tissue (0.70 ± 0.05-fold variation to control) when compared with the levels detected in the testicular tissue of rats fed *ad libitum* (Figure 5, Panel B). In addition, we also measured the levels of 4-HNE in testicular tissue of rats subjected to CR or fed *ad libitum*, but no differences were detected (Figure 5, Panel C). On the contrary, a decrease in 4-HNE was observed in spermatozoa of rats subjected to CR (0.78 ± 0.04-fold variation), when compared with the levels detected in the testicular tissue of rats fed *ad libitum* (Figure 5, Panel F). Finally, no differences were observed in the content of carbonyl and NT levels in spermatozoa of rats subjected to CR when compared with the levels detected in rats fed *ad libitum* (Figure 5, Panels D-E).

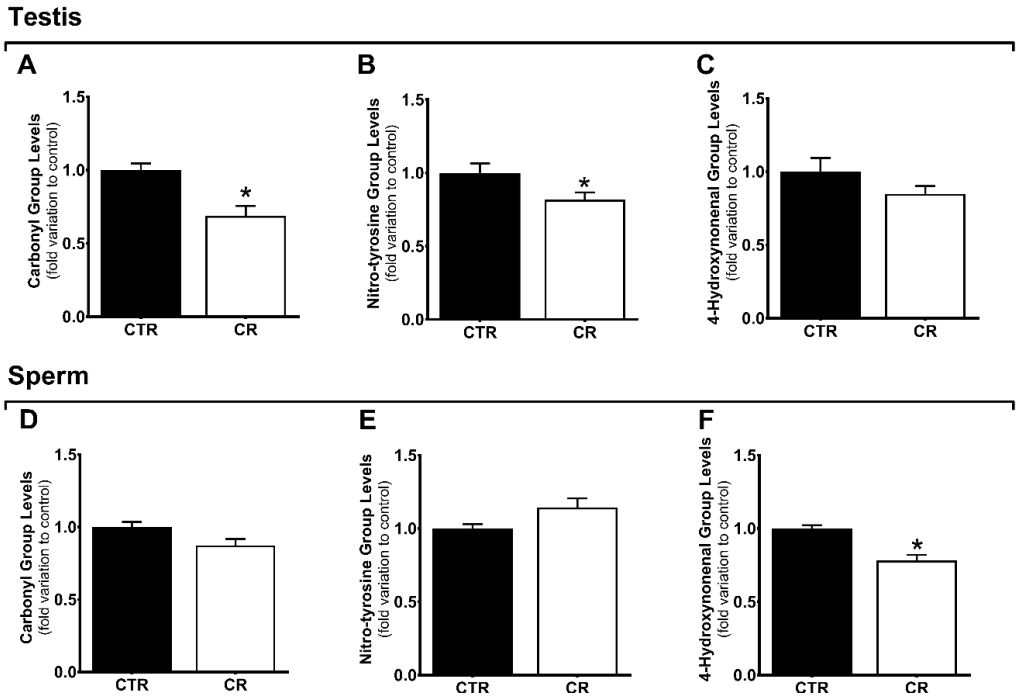

Figure 5. Effect of caloric restriction (CR) during 28 days in testicular tissue (Panel A-C) and spermatozoa (Panel D-F) oxidative damage compared with Wistar rats fed *ad libitum* (CTR). Panels A to F show pooled data of independent experiments, indicating the expression levels of carbonyl (Panel A-D), nitro-tyrosine (NT) (Panel B-E) and 4-hydroxynonenal (4-HNE) (Panel C and F). Panels A-F also show representative Slot blot experiments. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($p<0.05$) are as indicated: * relative to control.

Alterations in testicular metabolome induced by caloric restriction are not accompanied by changes in mTOR pathway

mTOR pathway has been involved in the response to energy deprivation caused by CR in several systems and organs [30]. In addition, recent reports highlighted a role for mTOR in testicular energy homeostasis [8, 31, 32]. Thus, we evaluated the expression levels of some intervenient of

mTOR pathway in the testicular tissue of rats subjected to CR and rats fed *ad libitum*. However, no changes were observed in the levels of S6 RP in the testicular tissue of rats subjected to CR when compared with the levels detected in the testis of rats fed *ad libitum* (Figure 6, Panel A). In addition, no statistically relevant changes in the levels of phosphorylated mTOR were observed in the testicular tissue of rats subjected to CR when compared with rats fed *ad libitum* (Figure 6, Panel B).

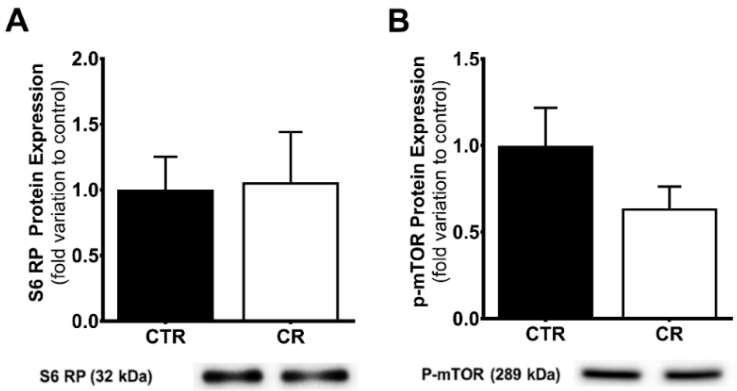

Figure 6. Effect of caloric restriction (CR) during 28 days in testicular tissue of Wistar rats compared with rats fed *ad libitum* (CTR) in mammalian target of rapamycin (mTOR) signaling pathway. The protein expression levels of S6 ribosomal protein (Panel A) and phosphorylated mTOR (Ser2448) (Panel B) in testicular tissue of Wistar rats subjected to a CR or fed *ad libitum* (CTR) are represented. Figure shows pooled data of independent experiments, indicating the expression levels of R6 ribosomal protein and P-mTOR. Figure also shows representative Western Blot. Results are expressed as mean ± SEM (n=6 for each condition).

Discussion

The impact of CR on male reproductive potential remains poorly investigated. Most particularly, whether CR influences the molecular mechanisms responsible for ensuring male reproductive physiological functions. A global study of gene expression profile in testis of mice subjected to a CR showed small effects on activation of testis-specific genes [33]. But, up to date, no data on the metabolic changes that occur in the testis of rats subjected to CR was available. Herein, we report the effects of CR on hormonal profile, overall testicular metabolic profile and sperm quality in a rat model. For that, male Wistar rats were fed for 28 days with 30% less standard chow diet than consumed by rats fed *ad libitum*.

As expected, rats subjected to CR had a lower body weight as previously observed in similar experimental protocols [34-36]. The hormonal profile is known to respond to the individual nutritional status, particularly leptin, ghrelin and GLP-1 plasma levels, which are directly involved in energy homeostasis regulation [37]. Indeed, a study by Collet and collaborators reported that the levels of these hormones are severely altered in individuals even after 48 hours of CR [38].

Similar results were obtained by analyzing the plasma of dogs subjected to a life-long CR [39] illustrating the importance of these hormones acting as energy sensors. In our study, rats subjected to CR had lower plasma leptin levels, a trend known to be associated with reduced food intake and/or body weight [38, 40-44]. Also, as expected, plasma levels of ghrelin in rats subjected to CR were higher, which is concurrent with leptin changes and is well documented to occur in response to hypocaloric diets [41, 44]. Finally, GLP-1 serum levels were found to be higher which was also observed in individuals under CR [41, 45]. This altered hormonal profile, associated with the biometric parameters, highlights the impact of CR on overall body energetic signaling. As energy balance is known to modulate spermatogenesis, we hypothesized that CR could alter testicular metabolome and change sperm quality.

In contrast to the well-recognized negative impact on female fertile potential [46, 47], the effect of CR on male fertility is often overlooked and is still a matter of intense debate. While some studies suggest that CR has no major effect on sperm quality [10, 48], others suggest that brief periods of CR or inefficient nutrition are still able to affect the efficiency of spermatogenesis [11, 12]. Our data demonstrate that a 28 days of CR diet induces deleterious effects on sperm morphology, particularly by inducing head defects. Higher levels of sperm head defects are related with lower fertility potential [49]. Indeed, higher number of sperm head defects are present in infertile individuals [49-51] and are also related with low pregnancy rates [52, 53]. We proposed that changes in sperm head morphology could be caused by metabolic changes induced by CR in testis. Although organs respond quite individually to CR, transcriptional analysis of both murine liver and muscle observed a switch from biosynthetic processes toward energy conservation [54]. Metabolomics analysis of plasma of non-human primates subjected to life-long CR suggests increased glucose flux through pentose phosphate pathway due to activation of peroxisome proliferator-activated receptor gamma (PPARγ) [55]. However, up to date there is no data on the global metabolic changes in testis under CR regime. Although, the general tendency for a decrease was observed for several amino acids (Glu, Pro, Ala, aspartic acid (Asp), branched-chain amino acids (BCAA)), the statistical significance was only achieved for the levels of Glu, Pro, and Ala. Decrease in the content of amino acids related to protein synthesis suggests decreased dietary availability of amino acids in CR group as well as decreased muscle production. Similar decrease in amino acid content was observed in the muscle of several sheep breeds exposed to seasonal weight loss [56] and in the systemic blood of non-human primates under CR [57]. Among those, BCAA act as regulators of protein turnover, by promoting protein synthesis and decreasing the rate of protein degradation through mTOR activation [58]. mTOR is also responsible for cell metabolism and ATP production [59]. Although no statistically relevant

changes in mTOR were observed in this study model, tendency toward decreased mTOR expression has been observed and can further explain the observed lower gonadal weight of the CR group. Nevertheless, our study further highlights that the role of mTOR signaling in mediating the effects of energy deprivation depends of species and CR duration. Further studies will be needed to test these hypotheses.

Lactate is indispensable for normal pachytene spermatocyte and spermatid metabolism and thus spermatogenesis [60]. Interestingly, testicular lactate levels, LDH activity and protein levels, as well as MCT4 expression, remained unaltered after CR illustrating that glycolysis appears to be sustained even after energy deprivation. The metabolic profile of murine testicular extracts is dominated by creatine [61], which is partly synthetized in Sertoli cells and is used in energy transport and storage by interstitial cells and developing sperm [62] as well as an oxidative stress-mediated mechanism. CR is associated with decrease of oxidative stress, however the effect is dependent on the duration of CR and is tissue specific [63]. Our study shows a decrease in carbonyl and NT substituted proteins, followed by an increase in transcript levels of *NRF1* in testicular tissue. These results are concomitant with other studies suggesting a decrease in oxidative damage in several tissues and organs of individuals subjected to CR. For instance, a study performed in 10 individuals with asthma demonstrated that a CR causes a decrease in serum levels of carbonyl and NT proteins compared with the measurement before the initiation of the intervention [64]. In male C57BL/6NNia mice, a decrease in carbonyl protein content was also found in brain, heart and kidney of mice subjected to CR [65]. In testis of rats subjected to a long term CR (40%, 6 months) a protective influence on age-related oxidative stress was detected, by increasing the levels of superoxide dismutase, glutathione peroxidase and catalase and decreased lipid oxidation [66]. However, endogenous antioxidants, like glutathione, ascorbate, choline or taurine, remained unaffected by the conditions of CR in our study. Apart from switching the preference of fuel source to the use of triglycerides, CR induced changes in other lipid classes as well, including phospholipids. In mice exposed to modest CR, favorable changes in the levels of arachidonic and its precursor linoleic acid were observed, suggesting decreased synthesis of lipid-derived inflammatory mediators in adipose tissue [67]. Jove and collaborators observed reduced levels of 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphatidylcholine, a specific marker of phospholipid oxidation in the liver of mice subjected to CR [68]. At the same time, the phospholipid signature was also affected, especially among phosphatidylethanolamines. The decrease in double bond number through decreased PUFA and increased MUFA suggested a remodeling in lipid membrane composition and was correlated with the reduced oxidative damage observed in phospholipids. Fuel adaptation under moderate CR also led to depletion of choline and

ethanolamine glycerophospholipid pools in murine myocardium [69] in a process followed by lipid membrane remodeling. Changes in choline and ethanolamine pools observed in our study indicate possible involvement of phospholipid metabolism in amelioration of testicular lipid oxidation observed in CR group.

This work proposed to study the effects of CR on hormonal profile and testicular metabolic profile and how it is reflected in sperm quality. Although 28 days CR had no direct effect on glucose metabolism of testicular tissue, it was possible to see a tendency in the levels of BCAA and other metabolites presenting two distinct profiles. In addition, our results show that CR decreases testicular oxidative markers, such as carbonyl and NT group levels. Notably, this was followed by a marked increase in spermatozoa head defects in rats subjected to CR. This is a very relevant parameter since it is associated with lower fertility potential and pregnancy rates. Taken together, our results show that CR alters testicular hormonal and metabolic profile and induces sperm head defects, which can compromise the fertility capacity of the males. Ultimately, our data highlights that CR can lead to subfertility or even infertility.

Supplemental Data

Table S1. Resonance assignment in the ^{1}H NMR spectra of polar testicular extracts

	Compound	δ ^{1}H ppm (multiplicity, assignment)
1	acetate[a]	1.90 (s, *α*CH_3)
2	adenosine/inosine	4.26 (m, ribose), 4.42 (m, ribose), 6.08 (d, C1'H ribose), 8.21 (s, C8H ring), 8.32 (s, C2H ring)
3	alanine	1.46 (d, *β*CH_3), 3.78 (t, *α*CH)
4	ascorbate	4.0 (m, C5H), 4.49 (d, C4H)
5	asparagine	2.84 (dd, *β*CH), 2.93 (dd, *β'*CH)
6	aspartate	2.67 (dd, *β*CH), 2.79 (dd, *β'*CH), 3.86 (m, *α*CH)
7	betaine	3.25 (s, CH_3), 3.89 (s, *α*CH_2)
8	choline	3.18 (s, CH_3), 3.50 (m, NCH_2), 4.04 (m, OCH_2)
9	citrate	2.54 (d, *α,γ*CH), 2.67 (d, *α ',γ'*CH)
10	creatine	3.02 (s, CH_3), 3.91 (s, CH_2)
11	cytidine	6.04 (d, C5H ring), 7.82 (d, C6H ring)
12	ethanolamine	3.12 (m, NCH_2), 3.82 (m, CH_2)
13	formate	8.46 (s, CH)
14	glutamate	2.03 (m, *β*CH), 2.11 (m, *β'*CH), 2.33 (m, *γ*CH_2), 3.74 (m, *α*CH)
15	glutamine	2.11 (m, *β*CH_2), 2.44 (m, *γ*CH_2)
16	glutathione	2.15 (q, *β*CH_2 Gl), 2.54 (m, γCH_2 Glu), 2.93 (m, *β*CH_2 Cys), 3.77 (m, *α*CH Gly), 4.56 (m, *α*CH_2 Cys)
17	glycerol	3.54 (dd, C1H_2), 3.63 (dd, C3H_2)
18	glycerophosphocholine	3.21 (s, CH_3), 3.67 (m, NCH_2), 4.30 (m, OCH_2)
19	glycine	3.54 (s, CH_2)
20	3-hydroxybutyrate	1.19 (d, *γ*CH_3), 2.28 (m, *α*CH), 2.39 (dd, *α*CH)
21	hypoxanthine	8.17 (s, C2H), 8.19 (s, C8H)
22	isoleucine	0.92 (t, *δ*CH_3), 0.99 (d, *β'*CH_3)
23	lactate	1.31 (d, *β*CH_3), 4.12 (q, *α*CH)
24	leucine	0.94 (t, *δ*CH_3), 1.69 (m, *βγ*CH_2)
25	lysine	1.71 (m, *δ*CH_2), 1.90 (m, *β*CH_2), 3.02 (m, *ε*CH_2)
27	Nicotinamide	7.58 (m, C5H ring), 8.24 (m, C4H ring), 8.69 (dd, C6H ring), 8.92 (dd, C2H ring)
28	phenylalanine	7.31 (m, C2H, C6H ring), 7.36 (m, C4H ring), 7.41 (m, C3H, C5H ring)
29	phosphocholine	3.20 (s, CH_3), 3.58 (m, NCH_2), 4.15 (m, OCH_2)
30	phosphoethanolamine	3.19 (M, NCH_2), 3.96 (m, OCH_2)
31	proline	1.99 (m, *γ*CH_2), 2.06 (m, *β'*CH), 3.32 (m, *β*CH, *δ´*CH_2), 3.4 (m, *δ*CH_2), 4.1 (m, *α*CH)
32	scyllo-inositole	3.35 (s, CH)
33	succinate	2.40 (s, CH_2)
34	taurine	3.24 (t, NCH_2), 3.41 (t, SCH_2)
35	threonine	1.31 (d, *γ*CH_3), 4.25 (m, *β*CH)
36	tyrosine	6.87 (d, C3H, C5H ring), 7.18 (d, C2H, C6H ring)
37	uracil	7.52 (d, C5H ring), 8.78 (d, C6H ring)
38	uridine	4.21 (m, ribose), 4.33 (m, C2'H ribose), 5.88 (d, C1'H ribose), 5.89 (d, C5H ring), 7.85 (d, C6H ring)
39	valine	0.97 (d, *γ*CH_3), 1.03 (d, *γ'*CH_3), 2.25 (m, *β*CH), 3.59 (m, *α*CH)
40	xanthine	7.9 (s, C2H ring)

(s: singlet, d: doublet, dd: doublet of doublets, t: triplet, m: multiplet).

[a] all metabolites are putatively annotated (level 2 of identification according to Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative recommendations)

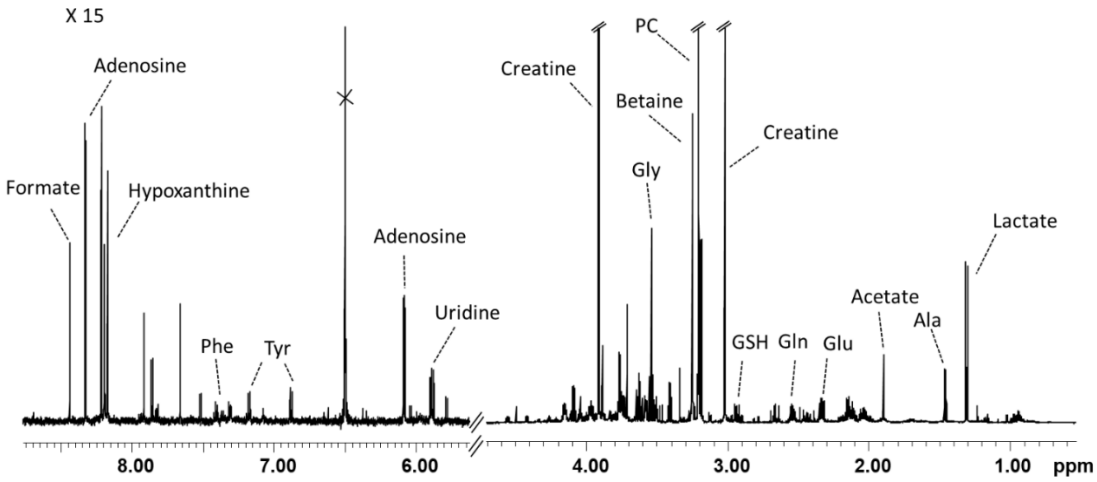

Figure S1: Typical ^{1}H NMR noesy spectrum of polar testicular extracts. Downfield part of the spectra (5.6-8.9 ppm) was vertically expanded 15x. Signals of some major metabolites are indicated: GSH glutathione, PC phosphocholine, three letter codes for amino acids.

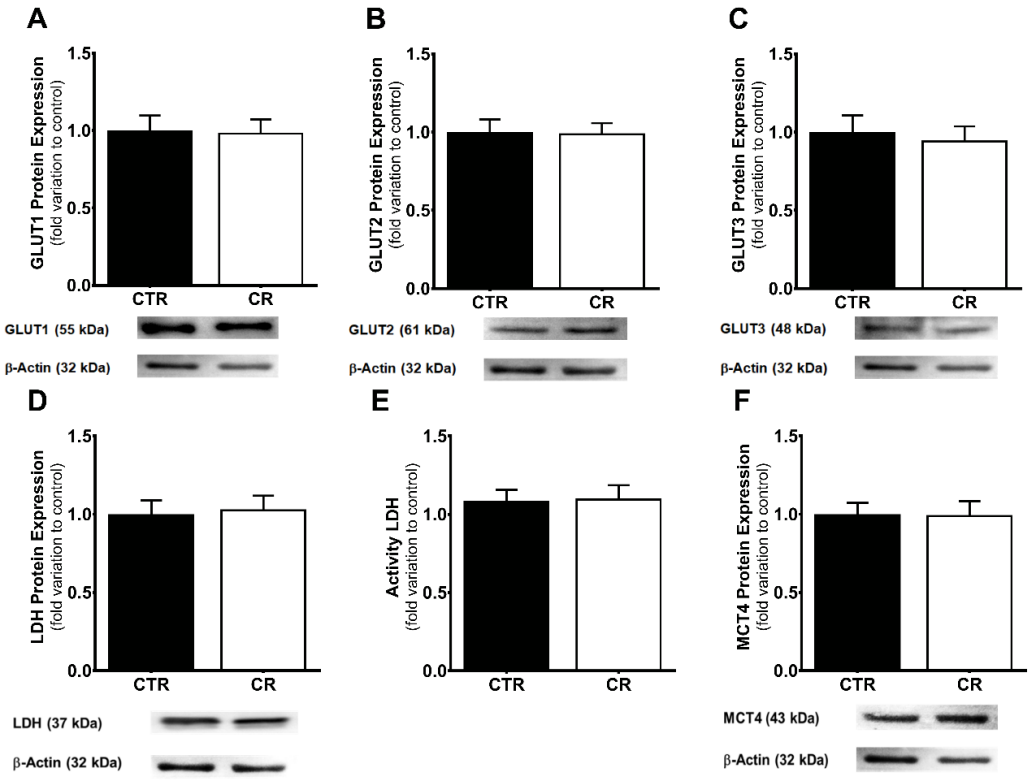

Figure S2: Effect of 28 days caloric restriction (CR) in Wistar rats testicular tissue in glucose metabolism related transporters and lactate dehydrogenase (LDH) compared with rats fed with a standard chow diet (CTR). Expression of glucose transporter 1 (GLUT1) (Panel A), glucose transporter 2 (GLUT2) (Panel B), glucose transporter 3 (GLUT3) (Panel C), LDH (Panel D), monocarboxylate acid transporter 4 (MCT4) (Panel F) and LDH activity (Panel E) are presented. Panels A to F show pooled data of independent experiments. Panels A-D and F also show representative Western Blot experiments. Results are expressed as mean ± SEM (n =6 for each condition).

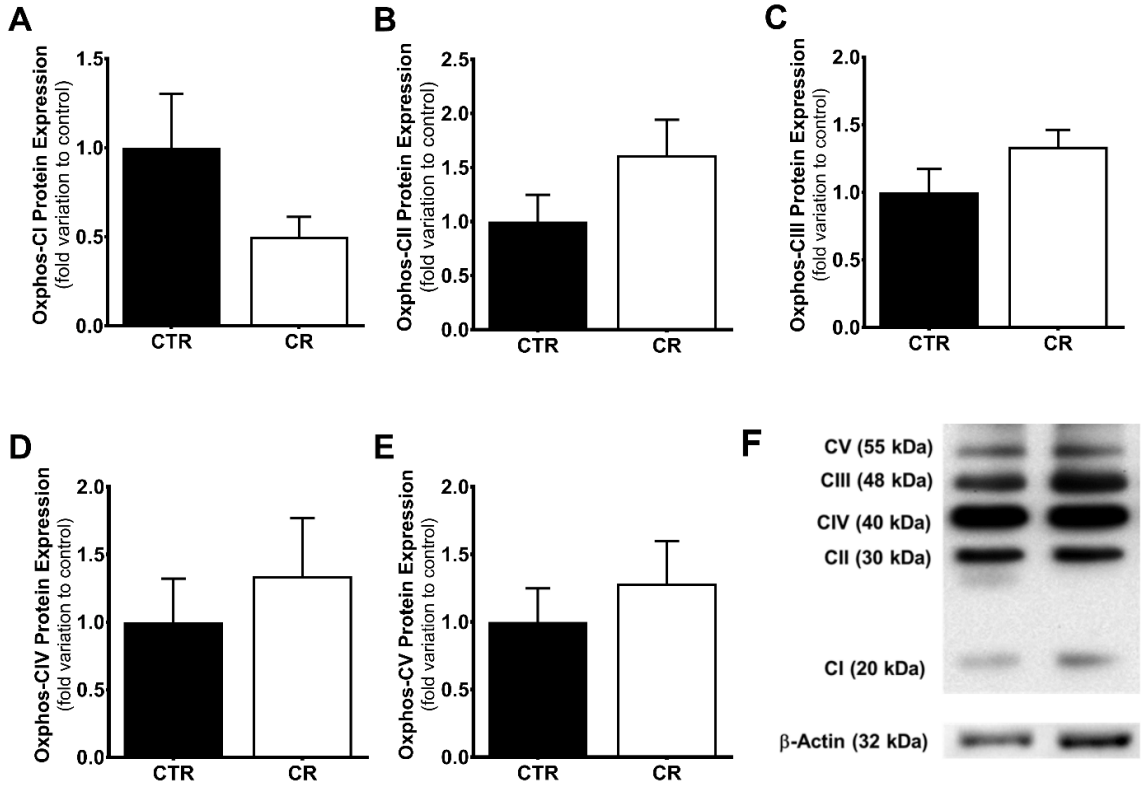

Figure S3: Effect of 28 days caloric restriction (CR) in testicular tissue mitochondrial complexes expression compared with Wistar rats fed with a normal diet (CTR). Expression of mitochondrial complex I (Panel A), complex II (Panel B), complex III (Panel C), complex IV (Panel D) and complex V (Panel E) is presented. Panels A to E show pooled data of independent experiments. Panels F also shows representative Western Blot experiments. Results are expressed as mean ± SEM (n =6 for each condition).

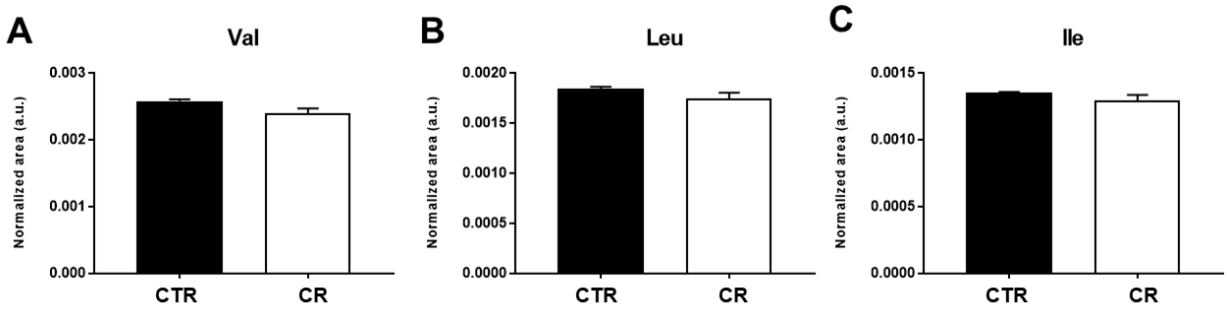

Figure S4: Effect of 28 days caloric restriction (CR) in testicular tissue content in valine (Val), leucine (Leu) and isoleucine (Ile) compared with Wistar rats fed with a normal diet (CTR). Statistical significance was evaluated by the t-test. Results were normalized by total area integral. Panels A-C show pooled data of independent experiments. Results are expressed as mean ± SEM (n=6 for each condition).

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Chapter 9

Concluding Remarks

Concluding Remarks

Infertility is a raising concern for several reasons, including the maintenance of generations and the genetic information we are passing. In Europe, the average of births *per* women is decreasing since 1950 to nowadays, from 3 to 1.6. In fact, in 2010 existing data shows that at least 50 million couples worldwide experience some type of infertility [1]. The infertility factor is divided by both partners in equivalent parts. In men, according to the International Classification of Diseases for Mortality and Morbidity Statistics, any disorder of the reproductive system, characterized by dysfunction in ejaculation or an abnormal absence in measurable levels of sperm can be classified as infertility [2, 3]. The causes for infertility can be of very distinct nature. In fact, the degradation of quality life is one of the main causes for the decrease in fertility rates we are witnessing in modern societies. Among the many factors, the exposure to harmful compounds, such as tobacco and alcohol are also relevant conditions affecting male fertility [4]. Also, a degradation of food habits and diet with a trend to substitute healthy food for high fat diets with low nutritional content which has a high impact in the population that tends to suffer from metabolic diseases, such as diabetes and obesity [5]. These diseases are well known to induce hormonal dysregulation including in leptin, ghrelin and glucagon like peptide-1 (GLP-1) in obesity [6] or insulin in diabetes [7].

Obesity is spreading worldwide and from 1975 to 2016, in low and middle -income countries, the rates almost tripled and in high-income countries is considered a problem [8, 9]. According to the World Health Organization (WHO), in 2016, more than 1.9 billion people with more than 18 years old were overweight and more than 650 million were already obese [9]. Many therapeutic options are available to treat obesity or diabetes. The therapy with glucagon like peptide-1 (GLP-1) and its analogues is one of the best options [10]. However, in response to an alarming increase in morbidly obese rates and its severity and impact in health care systems, bariatric surgery has become the most relevant option for obesity treatment in the last years [11]. In fact, in USA and worldwide, the number of surgeries has highly increased in the last years [12, 13]. Nevertheless, bariatric surgery does not excludes secondary and adverse effects [14]. After surgery there are several medical recommendations to the patient including a change in food habits [15]. However, sometimes after bariatric surgery the patients develop nutritional deficiencies [15, 16]. Thus, it is important to understand how nutritional deficiencies that cause hormonal dysregulation can affect male fertility.

The Sertoli cell has a crucial role in the development of the spermatozoa, being responsible for the nutritional support of the developing germ cells during the spermatogenesis. These cells are inside the testis and serve as major hormonal targets, being that it has been described that several hormones can regulate their metabolism [17-23]. Leptin and ghrelin are hormones responsible for the energy homeostasis together with GLP-1, during nutritional deficiencies and/or physiological states, however their effects on Sertoli cell and testicular metabolism were poorly studied.

To try to close the gap in the literature, this work aimed to study the modulation of human Sertoli cells metabolism and bioenergetics by leptin, ghrelin and GLP-1. These hormones are strongly dysregulated in conditions of poor food intake such as obesity or caloric restriction. Using an animal model, this work also aimed to elucidate the effects of a caloric restriction in testicular metabolism and sperm quality. The overall goal of this work was to study the hormonal regulation of spermatogenesis, particularly the role of ghrelin-leptin axis and GLP1 in nutrient membrane transport through Sertoli cell/ blood-testis barrier.

Leptin is mainly synthetized and secreted to the bloodstream by the adipose tissue, and blood leptin levels are directly correlated with the size of adipose tissue [24]. The circulating levels of leptin decrease in fasting conditions and in obesity or feeding conditions the levels increase [25]. In the first work, human cultured Sertoli cells were exposed to different concentrations of leptin for 24 hours and in a condition without exposure to the hormone. The concentrations were chosen based on the data available in the literature: 5 ng/mL was the physiological plasmatic levels found in lean, healthy patients [26] and the concentration found in seminal plasma [27]; 25 ng/mL was based on the plasmatic levels reported for obese patients [26]; 50 ng/mL the plasmatic levels found in morbidly obese men [28]. According to data attained, these cells presented a high glycolytic flux, through an increase in the expression of glucose transporter 2 (GLUT2) and lactate dehydrogenase (LDH) activity. A decrease in acetate was observed in a dose-response manner after exposure of the cells to leptin, which can be associated to the high glycolytic flux detected. The high flux of acetate produced by Sertoli cells is suggested to be crucial for the remodeling of the lipid membranes of the germ cells, though this hypothesis has not been already fully elucidated. We reported the expression of leptin receptor in Sertoli cells and the modulation of these cells metabolism by leptin in a way that the nutritional support of spermatogenesis can be compromised. This way, leptin levels known to occur in obese and morbidly obese men affects sperm production and consequently, it highlights a possible role for leptin in the establishment of the male fertility potential for these men.

Ghrelin is mainly secreted by a distinct group of enteroendocrine ghrelin cells localized in the gastric mucosa [29]. The levels of ghrelin in blood increase during prolonged fasting, while in

situations of food satisfaction there is a decrease [30, 31]. In this work, human Sertoli cells were exposed to different concentrations of ghrelin for 24 hours and also there was a condition where the cells were cultured without the compound. According to the literature we chose the plasmatic concentrations of: 100 pM that is found in normal weight healthy individuals [32, 33]; 20 pM that is frequently reported in obese individuals [31, 33]; and 500 pM that is reported in individuals under states of severe malnutrition [34, 35]. The exposure of Sertoli cells to 20 pM, which are the levels found in obese individuals, showed that lactate production was maintained but the cells consumed higher amounts of glucose and pyruvate, presenting a more oxidative profile. The oxidative status of Sertoli cells did not changed after exposure to ghrelin. In human Sertoli cells exposed to the high levels of ghrelin (500 pM), found in undernourished individuals, the cells presented a decrease in lactate production. Lactate is the preferred substrate for germ cell and has an anti-apoptotic effect, which means that when it is in non-physiological levels spermatogenesis is arrested and thus, male fertility compromised.

Glucagon like peptide-1 (GLP-1) is mainly produced by intestinal L-cells and secreted post-prandially [36] and has a role in glucose homeostasis predominantly mediated by the incretin effect. In this work, human cultured Sertoli cells were exposed to different concentrations of GLP-1 for 6 hours and cultured without exposure to GLP-1. The exposure time was chosen based on GLP-1 half-life [37]. The concentrations used were based in therapeutic doses of GLP-1 or analogues found in the literature: 0.01 nM is the postprandial GLP-1 plasmatic levels found in healthy individuals [38, 39] and the other doses were used to mimic the highest plasmatic concentrations achieved after administration of a GLP-1 analogue at the maximum therapeutic dosage recommended for obese individuals (3 mg/daily for liraglutide) either after a single administration (1 nM) or at steady state after a daily administration for 5 weeks (100 nM) [40-42]. The lactate production by human Sertoli cells exposed to 100 nM of GLP-1 was increased and was followed by a decrease in oxidative stress markers. Overall our data shows that GLP-1 analogues can positively affect the nutritional support of spermatogenesis thus, having a positive effect in overweight or obese men.

After bariatric surgery, patients are recommended to follow a strict diet, and nutrient deficiency are among one of the most serious adverse effects observed [15, 16]. In addition, there is a strong hormonal dysregulation in leptin, ghrelin and GLP-1 [43-45]. In order to try to mimic this condition we used an animal model subjected to caloric restriction. Wistar rats were randomly divided into two groups: one fed with normal diet (control) and other subjected to caloric restriction. Rats in the control group were fed *ad libitum* with a standard chow diet, while the rats subjected to caloric restriction received 30% less chow diet than the former for 28 days. Testicular tissue from rats

subjected to caloric restriction did not presented changes in glucose metabolism. The distinct metabolic profile when compared with control rats was mainly due to alterations in branched amino acids. Also, an increase in sperm head defects was observed in rats subjected to caloric restriction, a very important parameter associated with low pregnancy rates [46, 47]. This work highlights the relevance of the homeostasis on hormones associated with food intake as well as consumption of low calories.

In sum, our data supports that hormonal dysregulation on leptin and ghrelin in cases of overweight/obesity, followed by different therapeutic approaches, affect male fertility. Leptin and ghrelin in the levels found in obese individuals alter human Sertoli cells metabolism, particularly concerning acetate and lactate levels. The mechanism which by these hormones affect spermatogenesis is not clear yet, but these studies elucidate that the changes in these hormones alter the nutritional support of spermatogenesis and consequently change male fertility. Prolonged GLP-1 based therapies in obesity cases was mimic in cultured Sertoli cells and there was an increase in lactate production without inducing toxicity to the cells, which may counteract the negative effects of the dysregulation in ghrelin and leptin in the fertility of overweight or obese men. In addition to pharmacological therapies, caloric restriction can be a medical imposition or a consequence of bariatric surgery. Although our animal model does not show significant differences in testicular metabolome, spermatozoa from rats subjected to caloric restriction presented higher head defects, which is an important relevant for fertility. Taken together, our data clarifies some molecular mechanisms by which hormonal changes in leptin and ghrelin can affect Sertoli cell metabolism and consequently male fertility. We further elucidate the effects of GLP-1 and possible similar therapies in male fertility. Positive results were obtained showing that GLP-1 analogues can be useful to protect reproductive health of overweight/obese men. Finally, caloric restriction, and the hormonal dysregulation associated to it, has negative effects in testicular metabolism, sperm quality and consequently male fertility potential.

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Annexes

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Title: Ghrelin acts as energy status sensor of male reproduction by modulating Sertoli cells glycolytic metabolism and mitochondrial bioenergetics

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