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Effect of white tea on the reproductive function of diabetic or prediabetic individuals

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Dedication

For all the people who supported me every step of the way, in the university, in the laboratory, in daily life or at home. All the effort and dedication put into this research project made me grow professionally and personally. Thank you all.

"All our dreams can come true, if we have the courage to pursue them"
— Walt Disney

"I'm glad I did it, partly because it was worth it, but mostly because I shall never have to do it again"
— Mark Twain

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Preface

This document represents the completion of a research project to which I dedicated 7 years of my life. These were years of hard work that brought me a lot of knowledge and prepared me to pursue a career as a professional researcher. It was an ambitious project proposed by my supervisors that was overlooked by the research community, but once we published the first paper on the topic, an increasing interest on this subject emerged from many research groups. I am very proud of all the findings that resulted from our research, it was an incredibly rewarding journey. I am also honored for being able to work with wonderful people throughout these years. I hope this document and all our scientific publications on this topic constitute a step forward in the field of male reproduction.

"Science is telling us that we can do phenomenal things if we put our minds and our resources to it."

— Anthony S. Fauci

Resumo

A incidência da diabetes *mellitus* (DM) tem vindo a aumentar em homens jovens em todo o mundo. Os pacientes com DM apresentam uma disfunção na secreção de insulina e/ou na sua ação, resultando em hiperglicemia. Esta desregulação afeta a homeostase da glicose no organismo, tendo um impacto negativo na fertilidade masculina. As células de Sertoli (SCs) são essenciais para a manutenção do potencial reprodutivo masculino, pois são o suporte físico e nutricional que permite a diferenciação das células germinativas em espermatozoides totalmente competentes. A função das SCs depende do metabolismo da glicose, uma vez que este é o seu substrato preferencial. Muitos pacientes diabéticos são subférteis ou inférteis devido a alterações na função das SCs, defeitos na espermatogénese e má qualidade do esperma. A principal estratégia para combater a DM e as suas complicações inclui mudanças nutricionais e atividade física. No entanto, estas intervenções geralmente não são bem-sucedidas por si só e precisam de ser complementadas com medicação. Porém, para além da eficácia dos fármacos convencionais ser limitada, eles são caros e têm vários efeitos secundários. Nos últimos anos, os produtos naturais, incluindo o chá e os seus constituintes, têm vindo a demonstrar propriedades antioxidantes e antidiabéticas promissoras. Além disso, o tratamento da prediabetes pode constituir uma abordagem eficaz, pois esta condição pode ocorrer até 10 anos antes da progressão da doença para um estado mais grave. Neste projeto, pretendemos estudar os efeitos do chá branco (WTEA) na função reprodutiva masculina e o seu papel protetor contra disfunções reprodutivas induzidas pela prediabetes. Pretendemos ainda investigar se os efeitos do WTEA são devidos a um efeito combinado de todos os seus constituintes ou ao efeito predominante de um dos seus componentes bioativos mais representativos. O WTEA é o tipo de chá mais raro e menos estudado, mas apresenta um potencial antioxidante elevado, devido ao seu alto conteúdo em catequinas. Recorrendo à ressonância magnética nuclear de protão (^{1}H-NMR), verificou-se que este tipo de chá é particularmente rico em cafeína, epigalocatequina galato (EGCG) e L-teanina. Utilizando um modelo *in vitro* de SCs de rato ou humanas (hSCs) avaliámos os efeitos do extrato aquoso de WTEA (0.5 mg/mL) e dos seus principais componentes bioativos (cafeína, EGCG e L-teanina) no metabolismo celular, função mitocondrial e perfil oxidativo. O extrato de WTEA foi capaz de modular o metabolismo da glicose em SCs de rato e estimular a produção de lactato, um substrato essencial para a sobrevivência das células germinativas. A suplementação dos meios de cultura de hSCs com 50 μM de cafeína, EGCG ou L-teanina por 24 horas induziu alterações no metabolismo da glicose, importantes para melhorar o potencial reprodutivo masculino. Isto resultou num aumento ou manutenção da produção de lactato, mostrando um papel protetor contra os danos oxidativos. No entanto, na concentração de 50 μM, estes compostos também induziram algumas alterações na proliferação das hSCs e na sua função mitocondrial, o que pode comprometer a função reprodutiva. O extrato de WTEA (0.5 mg/mL) mostrou um melhor efeito na função das SCs do que os componentes individuais, destacando a importância da

combinação de todos os componentes do chá para o seu efeito benéfico. Foram também realizados estudos *ex vivo* utilizando espermatozoides epididimais de rato para avaliar o potencial do extrato de WTEA (0.5 mg/mL ou 1 mg/mL) e dos três componentes selecionados (cafeína, EGCG e L-teanina) como um aditivo para um meio de armazenamento de esperma à temperatura ambiente durante um curto período. Nestes estudos, a concentração da cafeína (71 µg/mL), EGCG (82 µg/mL) e L-teanina (19 µg/mL) foram estabelecidas de acordo com suas concentrações no extrato de WTEA. Salienta-se que a viabilidade dos espermatozoides diminui ao longo do tempo em amostras refrigeradas. Assim, em certos casos, o armazenamento a curto prazo à temperatura ambiente pode ser vantajoso, como por exemplo para o transporte de amostras ou tecnologia de reprodução medicamente assistida. Estes estudos permitiram concluir que o extrato de WTEA (principalmente na concentração de 1 mg/mL) é um melhor aditivo para o meio de armazenamento de espermatozoides à temperatura ambiente do que cada um dos componentes isoladamente, dado que manteve a viabilidade dos espermatozoides durante 3 dias de forma equivalente ao momento da colheita. Embora a combinação dos três componentes selecionados tenha mostrado uma melhoria na viabilidade dos espermatozoides relativamente a cada componente individualmente, também estimulou a oxidação das proteínas. Estes resultados apoiam também o benefício da combinação de todos os componentes do extrato de WTEA. Os dados obtidos nos estudos *in vitro* e *ex vivo*, levaram-nos a estudar o efeito *in vivo* do consumo regular de WTEA na função reprodutiva de um modelo animal de prediabetes. Os ratos prediabéticos mostraram alterações ao nível do metabolismo do testículo e do epidídimo, resultando numa diminuição da qualidade espermática. A ingestão de uma infusão de WTEA por ratos prediabéticos, durante dois meses, preveniu muitas das disfunções metabólicas induzidas pela doença, resultando na melhoria da motilidade e viabilidade espermática. Os nossos resultados indicam que o consumo regular de WTEA pode ser uma estratégia eficaz e de baixo custo para melhorar as disfunções reprodutivas induzidas pela prediabetes, abrindo caminho para que o WTEA possa vir a ser usado para o desenvolvimento de novas terapias antioxidantes para melhorar a fertilidade masculina.

Palavras-chave

Células de Sertoli; Chá branco; Diabetes *mellitus*; Fertilidade masculina; Metabolismo; Prediabetes; Qualidade espermática; Reprodução.

Resumo Alargado

A diabetes *mellitus* (DM) é uma doença metabólica tratável, mas geralmente incurável, caracterizada por hiperglicemia. Os indivíduos com esta condição apresentam defeitos no metabolismo das proteínas, gorduras e hidratos de carbono, principalmente devido a um mau funcionamento da secreção de insulina e/ou da sua ação. O número de diabéticos tem vindo a aumentar continuamente em todo o mundo. A *International Diabetes Federation* (IDF) estimou que até ao ano de 2035, o número de casos de DM pode chegar a aproximadamente 600 milhões de pessoas, afetando mais de um em cada 10 adultos no mundo. A diabetes tipo 2 (T2DM) representa 90-95% de todos os casos de DM. O seu desenvolvimento é um processo progressivo que ocorre quando se extingue a capacidade das células beta pancreáticas para compensar a incapacidade de as células do corpo usarem a insulina. A prediabetes é um estado intermédio caracterizado por níveis de glicose no sangue acima do normal, mas não altos o suficiente para ser considerado T2DM. Com as modificações certas no estilo de vida e medicação adequada, a prediabetes pode ser revertida ou controlada. Esta particularidade faz com que a investigação da prediabetes seja bastante importante, uma vez que pode levar a novas estratégias para retardar ou mesmo evitar o desenvolvimento da T2DM.

Nos últimos anos, os efeitos negativos da DM na fertilidade masculina têm sido amplamente investigados devido ao aumento da sua incidência em indivíduos mais jovens. Assim, a noção de que a DM é geralmente uma doença da população mais idosa foi desconsiderada. Atualmente, a DM afeta cada vez mais indivíduos antes e durante a idade reprodutiva, o que reforça a importância de estudar o seu impacto na reprodução masculina. De facto, vários estudos provam o declínio do potencial de fertilidade masculina nas últimas décadas. Por outro lado, o número de casais que procuram assistência médica para ter filhos tem vindo a aumentar. Aproximadamente 35% dos pacientes com T2DM são inférteis. Homens com T2DM ou prediabetes geralmente apresentam defeitos na espermatogénese e baixa qualidade espermática. As alterações hormonais e metabólicas induzidas pela doença são os principais contribuintes para esse cenário.

A primeira estratégia para combater a DM inclui mudanças nutricionais e atividade física. No entanto, essas intervenções geralmente não são bem-sucedidas por si só, sendo complementadas com medicação. Embora os fármacos convencionais sejam eficazes no controlo da glicemia, muitos têm efeitos adversos graves, como ganho de peso, hipoglicemia, edema e distúrbios gastrointestinais que podem desincentivar a adesão dos pacientes. Além disso, são bastante dispendiosos a longo prazo. Vários produtos naturais têm demonstrado um grande potencial antioxidante e antidiabético, destacando-se o chá e os seus componentes. O chá é uma das bebidas mais consumidas em todo o mundo e a sua popularidade está bastante associada aos seus efeitos benéficos para a saúde. Existem vários tipos de chá consoante a

colheira e o processamento das folhas. O chá branco (WTEA) é obtido através das folhas novas e rebentos da planta *Camellia sinensis*, os quais são minimamente processados de modo a evitar fermentação ou oxidação. Este tipo de chá destaca-se pelo seu potencial antioxidante elevado, principalmente devido ao seu alto conteúdo em catequinas. Neste projeto, pretendemos estudar os efeitos do WTEA na função reprodutiva masculina e o seu papel protetor contra disfunções reprodutivas induzidas pela prediabetes. Pretendemos ainda investigar se os efeitos do WTEA são devidos a um efeito combinado de todos os seus constituintes ou ao efeito predominante de um dos seus componentes bioativos mais representativos. Recorrendo à ressonância magnética nuclear de protão (^{1}H-NMR), verificou-se que este tipo de chá é particularmente rico em cafeína, epigalocatequina galato (EGCG) e L-teanina.

Utilizando um modelo *in vitro* de células de Sertoli de rato (SCs) ou humanas (hSCs) avaliámos os efeitos de um extrato aquoso de WTEA (0.5 mg/mL) e dos seus principais componentes bioativos (cafeína, EGCG e L-teanina) individualmente, no metabolismo celular, função mitocondrial e perfil oxidativo. As SCs são essenciais para a espermatogénese e, portanto, para a manutenção da fertilidade masculina. Estas células servem de suporte físico e nutricional para as células germinativas. O metabolismo da glicose é essencial para a função das SCs, uma vez que é o seu substrato preferencial. Estes estudos mostraram que o extrato de WTEA é capaz de modular o metabolismo da glicose em SCs de rato e estimular a produção de lactato, que é um substrato essencial para a sobrevivência das células germinativas. A suplementação dos meios de cultura de SCs humanas (hSCs) com 50 µM de cafeína, EGCG ou L-teanina por 24 horas induziu alterações no metabolismo da glicose que poderão ser importantes para melhorar o potencial reprodutivo masculino. Isto resultou num aumento ou manutenção da produção de lactato, mostrando um papel protetor contra os danos oxidativos a nível das proteínas e dos lípidos. No entanto, a cafeína (50 µM) diminuiu o potential antioxidante das hSCs e numa concentração mais elevada (500 µM) induziu um ambiente pro-oxidante. Por outro lado, a exposição das hSCs ao EGCG (50 µM) levou uma diminuição da proliferação das hSCs e do potencial da membrana mitocondrial, enquanto a exposição à L-teanina (50 µM) resultou num aumento desses parâmetros. Estas alterações podem comprometer a função das hSCs na função reprodutiva, levando-nos a concluir que o extrato de WTEA tem um efeito mais positivo na função das SCs, destacando a importância da combinação de todos os componentes do chá para o seu efeito benéfico.

Foram também realizados estudos *ex vivo* utilizando espermatozoides epididimais de rato para avaliar o potencial do extrato de WTEA (0.5 mg/mL ou 1 mg/mL) e dos três constituintes representativos selecionados (cafeína, EGCG e L-teanina) como um aditivo para um meio de armazenamento de esperma à temperatura ambiente durante um curto período. Nestes estudos, a concentração da cafeína (71 µg/mL), EGCG (82 µg/mL) e L-teanina (19 µg/mL) foram estabelecidas de acordo com suas concentrações no extrato de WTEA. Salienta-se que a viabilidade dos espermatozoides diminui ao longo do tempo quando as amostras são refrigeradas. Assim, em certos casos, o armazenamento a curto prazo à temperatura ambiente

pode ser vantajoso, como por exemplo para o transporte de amostras ou técnicas de reprodução assistida. Estes estudos permitiram concluir que o extrato de WTEA (principalmente na concentração de 1 mg/mL) foi um melhor aditivo para o meio de armazenamento de espermatozoides à temperatura ambiente do que cada um dos componentes isoladamente, dado que manteve a viabilidade dos espermatozoides durante 3 dias de forma equivalente ao momento da colheita. Embora a combinação dos três componentes selecionados tenha mostrado uma melhoria superior na viabilidade dos espermatozoides relativamente a cada componente individualmente, também estimulou a oxidação das proteínas. Estes resultados apoiam também o benefício da combinação de todos os componentes do extrato de WTEA.

No seguimento dos estudos anteriores, estudámos também o efeito *in vivo* do consumo regular de WTEA na função reprodutiva de um modelo animal de prediabetes. Os ratos prediabéticos mostraram alterações ao nível do metabolismo do testículo e do epidídimo, particularmente uma diminuição no conteúdo em lactato, resultando numa diminuição da qualidade espermática. A ingestão regular de WTEA por ratos prediabéticos durante dois meses preveniu muitas das disfunções metabólicas induzidas pela doença ao nível do testículo e epidídimo, resultando na melhoria da motilidade e viabilidade espermática. Os nossos resultados indicam que o consumo regular de WTEA pode ser uma estratégia eficaz e de baixo custo para melhorar as disfunções reprodutivas induzidas pela prediabetes, abrindo caminho para que o WTEA possa vir a ser usado para o desenvolvimento de novas terapias antioxidantes para melhorar a fertilidade masculina.

Abstract

The prevalence of diabetes *mellitus* (DM) has been increasing in young men worldwide. Patients with DM have a dysfunction on insulin secretion and/or insulin action, resulting in hyperglycemia. Insulin dysregulation affects glucose homeostasis in the body, having a deleterious impact on male fertility. Sertoli cells (SCs) are essential for the maintenance of male reproductive potential as they provide the physical and nutritional support that allows the differentiation of germ cells into fully competent spermatozoa. SCs function highly relies on glucose metabolism, which is their preferred substrate. Many diabetic patients are subfertile or infertile due to altered SCs function, impaired spermatogenesis and poor sperm quality. The primary strategy to counteract DM and its complications includes nutritional changes and physical activity. However, these interventions are usually unsuccessful alone and need to be complemented with medication. Still, the efficacy of conventional drugs is limited, they are expensive and have several secondary effects. In recent years, natural products, including tea and its components, demonstrated promising antioxidant and antidiabetic properties. Besides, the treatment of prediabetes can be an effective approach as it can occur up to 10 years before the progression of the disease to a more severe state. In this research project, we aimed to unravel the effects of white tea (WTEA) on male reproductive function and its protective role against reproductive dysfunctions induced by prediabetes. Further, we aimed to investigate if the effects of WTEA are due to a combined effect of all WTEA components or to a predominant effect of one of its most bioactive components. WTEA is the rarest and less studied type of tea, but it presents a potent antioxidant potential due to its high catechin content. Through proton nuclear magnetic resonance (^{1}H-NMR), we verified that this type of tea is particularly rich in caffeine, epigallocatechin gallate (EGCG) and L-theanine. Using an *in vitro* model of rat SCs or human SCs (hSCs) we evaluated the effects of WTEA extract (0.5 mg/mL) and its main bioactive compounds (caffeine, EGCG and L-theanine) on cells metabolism, mitochondrial functionality and oxidative profile. WTEA extract modulated rat SCs metabolism and stimulated the production of lactate, which is essential for germ cells survival. Supplementation of hSCs culture media with 50 µM of caffeine, EGCG, or L-theanine for 24 hours induced alterations in hSCs metabolism that are important for the improvement of male reproductive potential. It resulted in an increase or maintenance of lactate production, showing a protective role against oxidative damages. However, at 50 µM, these compounds also induced some alterations in hSCs proliferation and mitochondrial functionality that may compromise hSCs function. The WTEA extract (0.5 mg/mL) showed a better improvement on SCs, highlighting the importance of the combined effect of all the tea components for its beneficial effect. We also conducted *ex vivo* studies using rat epididymal spermatozoa to evaluate the potential of WTEA extract (0.5 mg/mL or 1 mg/mL) and the three selected components as an additive for a sperm storage medium at room temperature for short periods. In these studies, the concentrations of caffeine (71 µg/mL) EGCG (82 µg/mL) and L-theanine (19 µg/mL), were selected based on their concentration in

the WTEA extract. Sperm viability decreases over time in refrigerated samples, thus in certain cases the short-term storage at room temperature can be advantageous, such as for samples transport or assisted reproductive technology. These studies allowed us to conclude that the WTEA extract (especially at 1 mg/mL) was a better additive to the sperm storage medium at room temperature than each of the components alone, as it kept spermatozoa viability for 3 days equivalently to values obtained at the collection time. Although the combination of the three selected components together showed a higher improvement in spermatozoa viability, it also stimulated protein oxidation, supporting the beneficial combined effect of all the components constituting the WTEA extract. This led us to study the *in vivo* effect of a regular consumption of WTEA on the reproductive function of a rat model of prediabetes. The prediabetic rats showed alterations in the testicular and epididymal metabolism, resulting in poor sperm quality. WTEA ingestion by prediabetic rats for two months prevented many of the metabolic dysfunctions induced by the disease in the testis and epididymis, resulting in the improvement of sperm motility and viability. Our results indicate that WTEA regular consumption can be a cost-effective strategy to improve prediabetes-induced reproductive dysfunctions, paving the way for WTEA to be used for the development of new antioxidant therapies for the improvement of male fertility.

Keywords

Diabetes *mellitus*; Male fertility; Metabolism; Prediabetes; Reproduction; Sertoli cells; Sperm quality; White tea.

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List of Abbreviations

^{1}H-NMR	Proton nuclear magnetic resonance
4-HNE	4-hydroxynonenal
ABP	Androgen binding protein
ADP	Adenosine diphosphate
AI	Artificial insemination
ALT	Alanine transaminase
AMH	Anti-Müllerian hormone
AMP	Adenosine monophosphate
AP	Alkaline phosphatase
ART	Assisted reproductive technology
AT	Annealing temperature
ATP	Adenosine triphosphate
ATP5A	ATP synthase alpha-subunit
B2M	ß-2-microglobulin
BEB	Blood-epididymal barrier
BSA	Bovine serum albumin
BTB	Blood-testis barrier
cDNA	Complementary deoxyribonucleic acid
CPS	Counts per second
D_2O	Deuterium oxide
DM	Diabetes *mellitus*
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	2,4-dinitrophenol
DNPH	2,4-dinitrophenylhydrazine
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
EC	Epicatechin
ECG	Epicatechin gallate
EDTA	Ethylenediamine tetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ES	Ectoplasmic specialization
ETC	Electron transport chain
F12	Ham's F12 nutrient mixture
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GJs	Gap junctions
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GLUT3	Glucose transporter 3
GLUTs	Glucose transporters
GnRH	Gonadotropin releasing hormone
GRAS	Generally recognized as safe
GTEA	Green tea
GTT	Glucose tolerance test
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
HBSS	Hanks' balanced salt solution

HEPES	Hydroxyethyl piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
IDF	International Diabetes Federation
IGF-I	Insulin-like growth factor I
IGT	Impaired glucose tolerance
INT	3-(4-Iodophenyl)-2-(4-nitrophenyl)-5-phenyl-2H-tetrazol-3-ium chloride
IR	Insulin resistance
ITS	Insulin–transferrin–sodium selenite
ITT	Insulin tolerance test
IVF	In vitro fertilization
JC1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
LH	Luteinizing hormone
MARs	Matrix attachment regions
MCT4	Monocarboxylate transporter 4
MCTs	Monocarboxylate transporters
MDA	Malondialdehyde
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
M-PER	Mammalian protein extraction reagent
mRNA	Messenger ribonucleic acid
MTCO1	Mitochondrially encoded cytochrome c oxidase I
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD^+	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 8
ODF	Outer dense fibers
OS	Oxidative stress
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
PDH	Pyruvate dehydrogenase
PFK1	Phosphofructokinase 1
PMSF	Phenylmethylsulfonyl fluoride
PTM	Post-translational modifications
PUFAs	Polyunsaturated fatty acids
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SCs	Sertoli cells
SDHB	Succinate dehydrogenase complex, subunit B, iron sulfur
SP	Statistical power
SRB	Sulforhodamine B
STF	Seminiferous tubular fluid
STZ	Streptozotocin
T1DM	Type 1 diabetes *mellitus*
T2DM	Type 2 diabetes *mellitus*
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TJs	Tight junctions
TPTZ	2,4,6-tripyridyl-s-triazine
TYH	Krebs-Ringer bicarbonate
UQCRC2	Ubiquinol-cytochrome c reductase core protein II
VLDL	Very low-density lipoproteins
WHO	World Health Organization
WTEA	White tea

List of Publications

Publications included in this thesis:

Articles:

1 - Martins AD, Alves MG, Bernardino RL, **Dias TR**, Silva BM, Oliveira PF. (2013) "Effect of white tea (Camellia sinensis (L.)) extract in the glycolytic profile of Sertoli cell". European Journal of Nutrition 53(6):1383-1391 (DOI:10.1007/s00394-013-0640-5).

2 - Dias TR, Martins AD, Reis VP, Socorro S, Silva BM, Alves MG, Oliveira PF. (2013) "Glucose transport and metabolism in Sertoli cell: relevance for male fertility". Current Chemical Biology 7(3):282-293 (DOI:10.2174/22127968079991311281255 10).

3 - Dias TR, Tomás G, Teixeira N, Alves MG, Oliveira PF, Silva BM. (2013) "White tea (Camellia sinensis (L.): antioxidant properties and beneficial health effects". International Journal of Food Science, Nutrition and Dietetics 2(2):19-26 (DOI:dx.doi.org/10.19070/2326-3350-130005).

4 - Dias TR, Alves MG, Tomás GD, Socorro S, Silva BM, Oliveira PF. (2014) "White tea as a promising antioxidant media additive for sperm storage at room temperature: a comparative study with green tea". Journal of Agricultural and Food Chemistry 62(3): 608–617 (DOI:10.1021/jf4049462).

5 - Dias TR, Alves MG, Silva BM, Oliveira PF. (2014) "Sperm glucose transport and metabolism in diabetic individuals". Molecular and Cellular Endocrinology 396(1-2):37-45 (DOI:10.1016/j.mce.2014.08.005).

6 - Dias TR, Alves MG, Oliveira PF, Silva BM. (2014) "Natural products as modulators of spermatogenesis: the search for a male contraceptive". Current Molecular Pharmacology 7(2):154-166 (DOI:10.2174/1874467208666150126155912).

7 - Alves MG, **Dias TR**, Silva BM, Oliveira PF. (2014) "Metabolic cooperation in testis as a pharmacological target: from disease to contraception". Current Molecular Pharmacology 7(2):83-95 (DOI:10.2174/1874467208666150126153830).

8 - Dias TR, Alves MG, Bernardino RL, Martins AD, Moreira AC, Silva J, Barros A, Sousa M, Silva BM, Oliveira PF. (2015) "Dose-dependent effects of caffeine in human Sertoli cells metabolism and oxidative profile". Toxicology 328:12-20 (DOI:10.1016/j.tox.2014.12.003).

9 - Dias TR, Alves MG, Casal S, Silva BM, Oliveira PF. (2016) "Single and synergistic effect of major tea components caffeine, epigallocatechin gallate and L-theanine in rat sperm viability". Food & Function 7(3):1301-1305 (DOI:10.1039/c5fo01611h).

10 - Dias TR, Alves MG, Rato L, Casal S, Silva BM, Oliveira PF. (2016) "White tea intake prevents prediabetes-induced metabolic dysfunctions in testis and epididymis preserving sperm quality" The Journal of Nutritional Biochemistry 37:83-93 (DOI:10.1016/j.jnutbio.2016.07.018).

11 - Dias TR, Bernardino RL, Meneses MJ, Sousa M, Sá R, Alves MG, Silva BM, Oliveira PF. (2016) "Emerging potential of natural products as an alternative strategy to pharmacological agents used against metabolic disorders". Current Drug Metabolism 17(6):582-597 (DOI:10.2174/1389200217666160229113629).

12 - Dias TR, Alves MG, Silva J, Barros A, Sousa M, Casal S, Silva BM, Oliveira PF. (2017) "Implications of epigallocatechin gallate in cultured human Sertoli cells glycolytic and oxidative profile" Toxicology In Vitro 41:214-222 (DOI:10.1016/j.tiv.2017.03.006).

13 - Dias TR, Alves MG, Casal S, Oliveira PF, Silva BM. (2017) "Promising potential of dietary (poly)phenolic compounds in the prevention and treatment of diabetes *mellitus*" Current Medicinal Chemistry 24(4):334-354 (DOI:10.2174/0929867323666160905150419).

14 - Dias TR, Alves MG, Oliveira PF, Silva BM. (2017) "Bioactive Substances from Medicinal Plants for Metabolic Disorders" Current Medicinal Chemistry 24(4):332-333 (DOI:10.2174/0929867324041703022233350).

15 - Dias TR, Bernardino RL, Alves MG, Silva J, Barros A, Sousa M, Casal S, Silva BM, Oliveira PF. (2019) "L-theanine promotes cultured human Sertoli cells proliferation and modulates glucose metabolism" European Journal of Nutrition 58(7):2961-2970 (DOI:10.1007/s00394-019-01999-2).

Book Chapters:

16 - Dias TR, Alves MG, Neuhaus-Oliveira A, Socorro S, Silva BM, Oliveira PF. (2013) "Implications of Diabetes on sperm glucose uptake and metabolism" In Glucose Uptake: Regulation, Signaling Pathways and Health Implications, Nova Science Publishers, Inc, New York, USA, pp. 141-168 (ISBN: 978-1-62618-671-2).

17 - Dias TR, Bernardino RL. (2017) "Biochemical events occurring in the epididymis" In: Alves MG, Oliveira PF (eds) Biochemistry of Andrology, vol 1. Andrology: Current and Future Developments. Bentham Science Publishers, Sharjah, UAE, pp. 178-206 (DOI: 10.2174/97816810850051170101), (ISBN:978-1-68108-501-2).

18 - Dias TR. (2017) "Functional and biochemical aspects of spermatozoa" In: Alves MG, Oliveira PF (eds) Biochemistry of Andrology, vol 1. Andrology: Current and Future Developments. Bentham Science Publishers, Sharjah, UAE, pp. 230-256 (DOI: 10.2174/97816810850051170101), (ISBN:978-1-68108-501-2).

19 - Dias TR, Alves MG, Silva BM, Oliveira PF. (2018) "Nutritional factors and male reproduction" In: Bernard Jégou and Michael K. Skinner (Eds), Encyclopedia of Reproduction, 2nd edition, volume 1, Elsevier, Waltham, MA, USA, pp. 458-464 (DOI:10.1016/B978-0-12-801238-3.64616-0), (ISBN:978-0-12-801238-3).

20 - Dias TR, Carrageta DF, Alves MG, Oliveira PF, Silva BM. (2018) "White tea" In: Seyed Nabavi and Ana Silva (eds), Nonvitamin and Nonmineral Nutritional Supplements, 1st edition, Academic Press, Elsevier, pp. 437-445 (DOI: 10.1016/B978-0-12-812491-8.00058-8), (ISBN:9780128124918).

Chapter 1

Introduction

This chapter includes the following sections:

- Male reproductive biology and physiology
- Diabetes *mellitus* and male fertility
- Nutrition, antioxidants and male reproduction

Male reproductive biology and physiology

This section was adapted from the following publications:

- **Dias TR**, Martins AD, Reis VP, Socorro S, Silva BM, Alves MG, Oliveira PF. (2013) "Glucose transport and metabolism in Sertoli cell: relevance for male fertility". Current Chemical Biology 7(3):282-293 (DOI:10.2174/22127968079991311281255l0).

- **Dias TR**, Alves MG, Oliveira PF, Silva BM. (2014) "Natural products as modulators of spermatogenesis: the search for a male contraceptive". Current Molecular Pharmacology 7(2):154-166 (DOI:10.2174/1874467208666150126155912).

- Alves MG, **Dias TR**, Silva BM, Oliveira PF. (2014) "Metabolic cooperation in testis as a pharmacological target: from disease to contraception". Current Molecular Pharmacology 7(2):83-95 (DOI:10.2174/1874467208666150126153830).

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- **Dias TR**. (2017) "Functional and biochemical aspects of spermatozoa" In: Alves MG, Oliveira PF (eds) Biochemistry of Andrology, vol 1. Andrology: Current and Future Developments. Bentham Science Publishers, Sharjah, UAE, pp. 230-256 (DOI: 10.2174/97816810850051170101), (ISBN:978-1-68108-501-2).

Spermatogenesis

In mammals, about 80% of the testicular mass consists of highly coiled seminiferous tubules [1, 2], while the remaining 20% are Leydig cells and other interstitial components [1]. Spermatogenesis takes place in the seminiferous tubules, of which the main structural elements are Sertoli cells (SCs) [3]. Spermatogenesis refers to the development of mature spermatozoa with half the number of chromosomes (haploid), from the most immature germ cell in the testis, spermatogonium (diploid) [4, 5]. This complex process is divided in three major phases: mitotic, meiotic and spermiogenesis [6]. Firstly, undifferentiated spermatogonia, located along the basement membrane of the seminiferous tubules can be of two types: with dark (A_d) or pale (A_p) nuclei [7]. A_d cells do not present active mitosis, thus constituting the reserve of spermatogonial stem cells that allow the maintenance of spermatogenesis from puberty until death. This explains why despite aging in males is associated with a decline in semen volume, sperm motility and normal morphology, sperm concentration is not altered with aging [8]. On the other hand, A_p cells are the spermatogonial steam cells that differentiate into type B spermatogonia, which then migrate closer to the tubule lumen and differentiate into slightly larger cells called primary spermatocytes (diploid) [7]. In the second phase, primary spermatocytes move into the adluminal compartment of the seminiferous tubules and divide into two secondary spermatocytes (haploid) by meiosis I. Then, each secondary spermatocyte undergo meiosis II resulting in two haploid round spermatids [9]. Finally, spermiogenesis involves the transformation of round spermatids into elongated flagellar spermatids and culminates with the release of spermatozoa (spermiation) into the lumen of the seminiferous tubules [9, 10]. During spermiogenesis, spermatids suffer a number of morphological changes including acrosome formation, nuclear condensation, development of the flagellum, and cytoplasm reorganization, which eventually result in the generation of spermatozoa [9]. Those sequential alterations in the spermatids differentiation are very helpful in the identification of the stages of the spermatogenic cycle [11]. In most mammals, each spermatogenic cycle takes 9-12 days. In the mouse takes about 8.6 days (XII stages) and in the rat 12.9 days (XIV stages) [11]. However, the human spermatogenic cycle is longer, lasting 16 days (VI stages) and four cycles are needed to complete spermatogenesis (more than 70 days) [12].

Spermatogenesis is under a strict hormonal control driven by the communication between the hypothalamus–pituitary axis and the gonad itself (Figure 1.1) [13]. The master regulator of this process is the gonadotropin releasing hormone (GnRH) [14], which triggers the release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary (Figure 1.1) [15]. LH binds to receptors on the surface of Leydig cells stimulating the production of testosterone, a steroid hormone that diffuses into the seminiferous tubules. Within the seminiferous tubules, only SCs possess receptors for testosterone and FSH [14]. FSH stimulates SCs division and differentiation, as well as the production of androgen-binding protein (ABP), facilitating the passage of testosterone through the blood-testis barrier (BTB). Thus,

testosterone and FSH act synergistically on SCs leading to the secretion of paracrine agents that are essential for spermatogenesis [13]. SCs convert testosterone into estrogen and the circulating levels of these two hormones mediate the feedback inhibition of GnRH through steroid receptors present in the hypothalamic neurons and in the pituitary [16]. SCs also secrete the hormones inhibin and activin that also regulate GnRH and LH/FSH release.

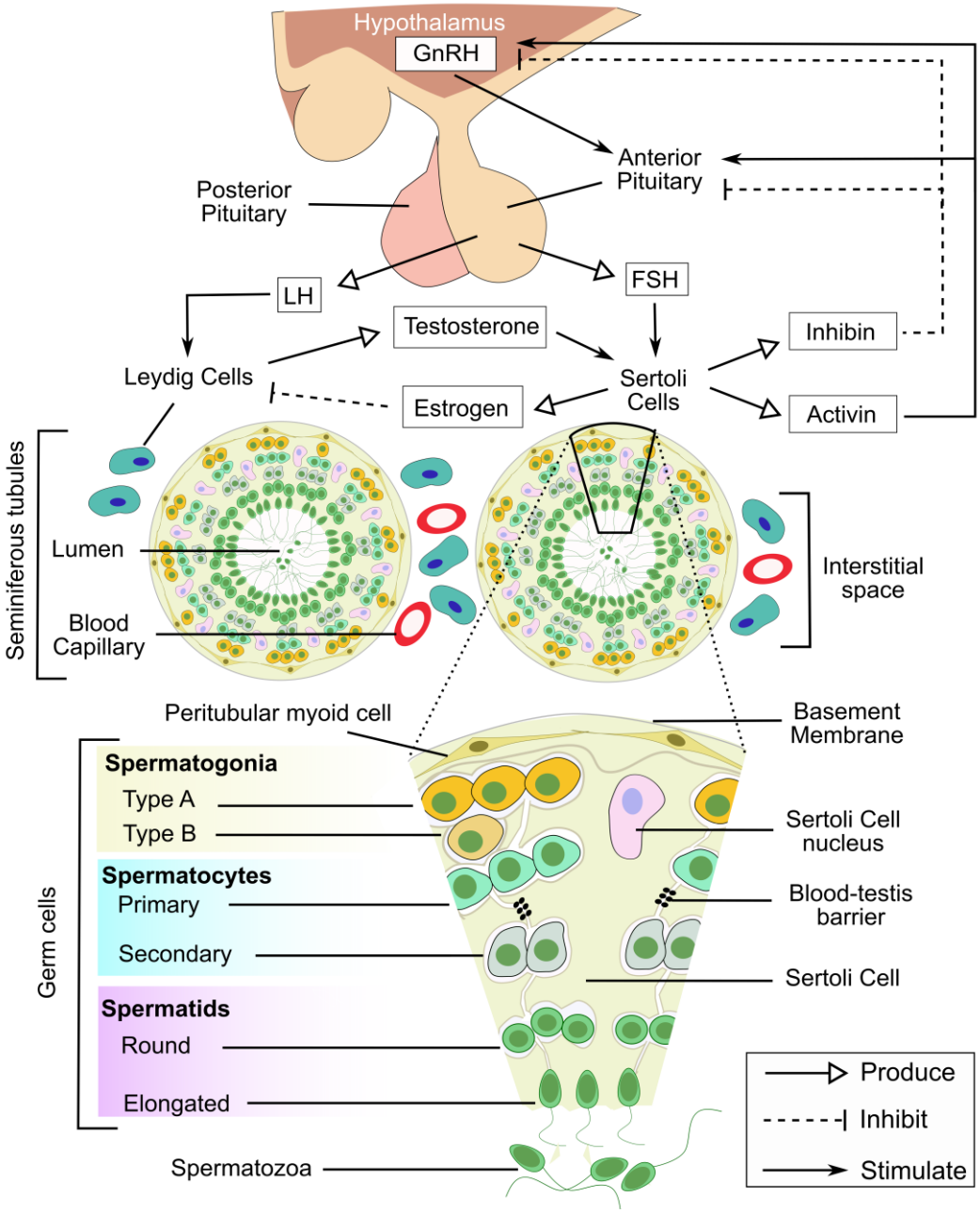

Figure 1.1 Spermatogenesis and its hormonal control. Spermatogonia divides by mitosis originating primary spermatocytes, which transform into secondary spermatocytes after the first meiotic division. Then, meiosis II give rise to round spermatids, which subsequently differentiate into elongated spermatids. The process culminates with the release of spermatozoa into the lumen of the seminiferous tubule (spermiation). Spermatogenesis is mainly regulated by the gonadotropin releasing hormone (GnRH), synthetized by the hypothalamus, which stimulates the anterior pituitary to produce the luteinizing hormone (LH) and follicle-stimulating hormone (FSH). FSH acts only on the Sertoli cells (SCs), present in the seminiferous tubules, stimulating spermatogenesis. SCs secrete hormones inhibin and activin, which regulate GnRH and LH/FSH release. LH acts only on the Leydig cells inducing testosterone production. SCs convert testosterone into estrogen, which has an inhibitory effect on Leydig cells androgen production.

Sertoli cells

The SCs are highly polarized epithelial cells that extend upwards from the basement membrane, where the peritubular myoid cells reside, towards the lumen of the seminiferous tubules, surrounding the germ cells (Figure 1.1) [17, 18]. SCs play a central role in the development of a functional testis, and hence in the expression of the male phenotype [19]. Testis determination in mammals occurs through the action of the Y-linked gene *Sry*. The *Sry* gene encoded protein acts in the supporting cell lineage of the indifferent gonad, triggering a cascade of events that results in the differentiation of these cells into Sertoli rather than follicle cells [20]. Then, SCs undergo a phase of rapid cell proliferation and differentiation [21] activating other cell lineages within the gonad to follow the testicular pathway [20]. At each division of pre-pubertal SCs, the daughter cells generate specialized micro-domains to sustain the amplification of the mitotic spermatogonia [22]. The anti-Müllerian hormone (AMH) is the first product secreted by SCs in the developing testes [23], ensuring the regression of the Mullerian ducts, which is a crucial step for proper male sexual differentiation [24].

Around the onset of puberty, the final phase of SCs differentiation is marked by the cessation of proliferation and irreversible changes in SCs morphology and physiology [18], heralding the switch from an immature, proliferative state to a mature, non-proliferative state [25]. In this terminal differentiation phase, SCs exit from the cell cycle and originate the BTB [21], marking the progressive entry of SCs into adulthood. Besides, the lumen of the tubules is formed and SCs acquire the typical adult characteristics: the nucleus, usually situated in the basal portion of the cell, enlarges and becomes tripartite; the nucleolus becomes more prominent [25]; the cytoplasm becomes voluminous and include cytoplasmic components with a polar distribution; the organelles and inclusions appear in higher amounts in the basal and lower trunk of the cell, whereas mitochondria and smooth endoplasmic reticulum are more abundant in the apical portion [26]. SCs also contain a relatively low proportion of rough endoplasmic reticulum, an elaborate cytoskeleton, lipids and lysosomes [18]. SCs differentiation is also accompanied by the expression of many gene products that are not present in immature cells [27-32]. Nevertheless, there is an interesting feature about adult SCs proliferation. Although it has been reported that SCs cease to divide and proliferate at a certain phase of their adulthood, being that time different between species [25], this affirmation revealed not to be an absolute true. When adult SCs were transplanted into impaired or defective testis, were found to be proliferative, capable of populating the testis and even restoring spermatogenesis [33, 34]. Moreover, several *in vitro* studies have shown that this cellular type has a proliferative potential when cultured in favorable conditions [35, 36]. In fact, an *in vitro* model of adult SCs retain many specific characteristics of their derived tissues, being a great model for *in vitro* toxicology studies [37].

The BTB is one of the tightest blood-tissue barriers in mammals [2]. It is formed by coexisting specialized junctions between adjacent SCs, including tight junctions (TJs), basal ectoplasmic

specialization (ES), gap junctions (GJs) and desmosome-like junctions [38-42], such that nothing larger than 1000 dalton can pass from the outside to the inside of the tubule [14, 43]. The BTB divides the seminiferous epithelium into the basal compartment (containing spermatogonia and early spermatocytes) and adluminal (or apical) compartment (containing spermatocytes in different stages of differentiation, spermatids and spermatozoa) [2, 43]. This barrier consists in three components: i) an anatomical/physical barrier to restrict the entry of molecules into the adluminal compartment; ii) an immunological barrier that limits the movement of immune cells and regulates the level of cytokines in the seminiferous epithelium; and iii) a physiological barrier that is highly dynamic to encounter the needs of germ cells [1, 44]. These components create a special microenvironment responsible for the proper development of spermatogenesis. Particularly, BTB allows the control of the ionic composition and pH of the seminiferous tubular fluid (STF) [35, 45, 46]. Thus, BTB dysfunction may lead to the arrest of germ cells differentiation [47, 48].

Sertoli-germ cells cooperation

Germ cells development is a highly organized process that is mainly under the control of SCs [49]. It has been established that each SC can only support a limited number of germ cells (30 to 50) [50], and approximately 75% of developing germ cells undergo spontaneous degeneration [51]. This will determine the daily sperm production, a factor that will impact male fertilizing potential [25, 52]. Mature SCs acquire a characteristic spatial arrangement that provides them the unique capability to interact either morphologically and/or chemically with the different generations of germ cells, with peritubular myoid cells and with the steroid producing Leydig cells. The SCs base is in contact with spermatogonia, while their lateral surfaces send processes around spermatocytes and early spermatids; their apical portion is intimately associated to elongating and elongated spermatids and faces the tubule lumen where spermatozoa are released (Figure 1.1) [18]. There is a well-timed movement of developing germ cells across the BTB consisting of intermittent phases of junctional complexes disassembly and reassembly, which allow the passage of germ cells while maintaining BTB integrity [53]. In the beginning of meiosis, germ cells located outside the barrier pass through the TJs and, once beyond the BTB, they are dependent on SCs supplies [43]. If the passage across BTB is accelerated, this will induce a premature detachment of germ cells from the epithelium and the spermatozoa produced are unable to fertilize the egg due to their immaturity. Likewise, if the process is hampered and germ cells become retained in the epithelium, they will be removed by SCs via phagocytosis. In either case, subfertility or infertility may occur [53], highlighting the importance of a proper SCs function.

In 1865, Enrico Sertoli gave his name to SCs and defined them as “nurse cells” [54]. With this concept, he meant that SCs provide nutrients, regulatory factors, functional glycoproteins and peptides for the development and differentiation of germ cells [55-58]. Particularly, SCs

provide factors required to fuel germ cells metabolism (lactate, transferrin, ceruloplasmin, ABP) [59, 60]; growth regulatory factors (stem cell factor, transforming growth factors alpha and beta - TGF-α and TGF-β); insulin-like growth factor-I (IGF-I); fibroblast growth factor (FGF); and epidermal growth factor (EGF) [60]. SCs have the ability to metabolize various substrates, but preferentially use glucose [61, 62], even though this monosaccharide (hexose) is present at very low levels in STF due to its rapid metabolism [62]. Glucose transport through SCs cytoplasmic membrane is mediated by glucose transporters (GLUTs), particularly GLUT1, GLUT2 and GLUT3 (Figure 1.2) [63]. Glucose enters the glycolytic pathway and is decomposed to pyruvate, in a rate-limiting process catalyzed by the enzyme phosphofructokinase 1 (PFK1) [64]. This cascade of reactions yields not only two molecules of pyruvate, but also two molecules of adenosine triphosphate (ATP) and two electron-carrying molecules of nicotinamide adenine dinucleotide reduced (NADH) [62, 65]. Under low-oxygen conditions, glycolysis end-products follow into the production of lactic acid, by lactate dehydrogenase (LDH) action [66]. LDH is responsible for the reversible conversion of pyruvate into lactate, with the concomitant oxidation/reduction of NADH to its oxidized form NAD^+ [67]. After lactate production, it is crucial that this product becomes available for the developing germ cells, as it is their preferred substrate for ATP production [60, 68-70]. This event is mediated by active membrane monocarboxylate transporters (MCTs), especially MCT4, that are responsible for lactate transport through the plasma membrane of SCs [63, 71-73] (Figure 1.2). The importance of lactate for normal spermatogenesis is highlighted in a report showing that spermatogenesis in adult cryptorchid testis is improved by intratesticular infusion of lactate [74]. It is well known that spermatogonia may utilize glucose as the major energy substrate [75], but spermatocytes and spermatids suffer a rapid decline in their ATP content in glucose-supplemented media, thus, they require lactate/pyruvate for the maintenance of their ATP concentrations [76, 77]. Interestingly, spermatozoa prefer glucose/fructose as energy source [78]. Nevertheless, an although the regulatory molecular mechanisms by which SCs preferentially export lactate, pyruvate or glucose at each stage of spermatogenesis remain largely unknown [79], it is widely accepted that glucose and its metabolites play a pivotal role at many stages of germ cells development [80].

Pyruvate is an important regulatory point of SCs metabolism as it can follow two other pathways besides lactate production: it can be converted to alanine in a reversible reaction catalyzed by alanine transaminase (ALT) [81], or it can enter the mitochondria to originate acetyl-CoA by the action of pyruvate dehydrogenase (PDH) [82]. Alanine plays a key role in the maintenance of cellular redox status and glucose homeostasis [9]. In fact, the interconversion lactate – pyruvate - alanine is NADH-dependent and the $NADH/NAD^+$ ratio can be estimated by the ratio lactate/alanine, which is often used as a measure of the cellular redox state [83]. On the other hand, acetyl-CoA can enter the Krebs cycle, where it is converted to citrate [84], or it can be exported to the cytosol and form acetate [85], which can be used for fatty acids and cholesterol synthesis [86]. When there is a Krebs cycle truncation, citrate can also be transported to the

cytosol or to extracellular compartment [84]. Under physiological conditions, glucose metabolism is strictly controlled and is essential for a normal spermatogenesis [10]. However, the dysregulation of glucose metabolism may lead to male reproductive disorders [11, 12].

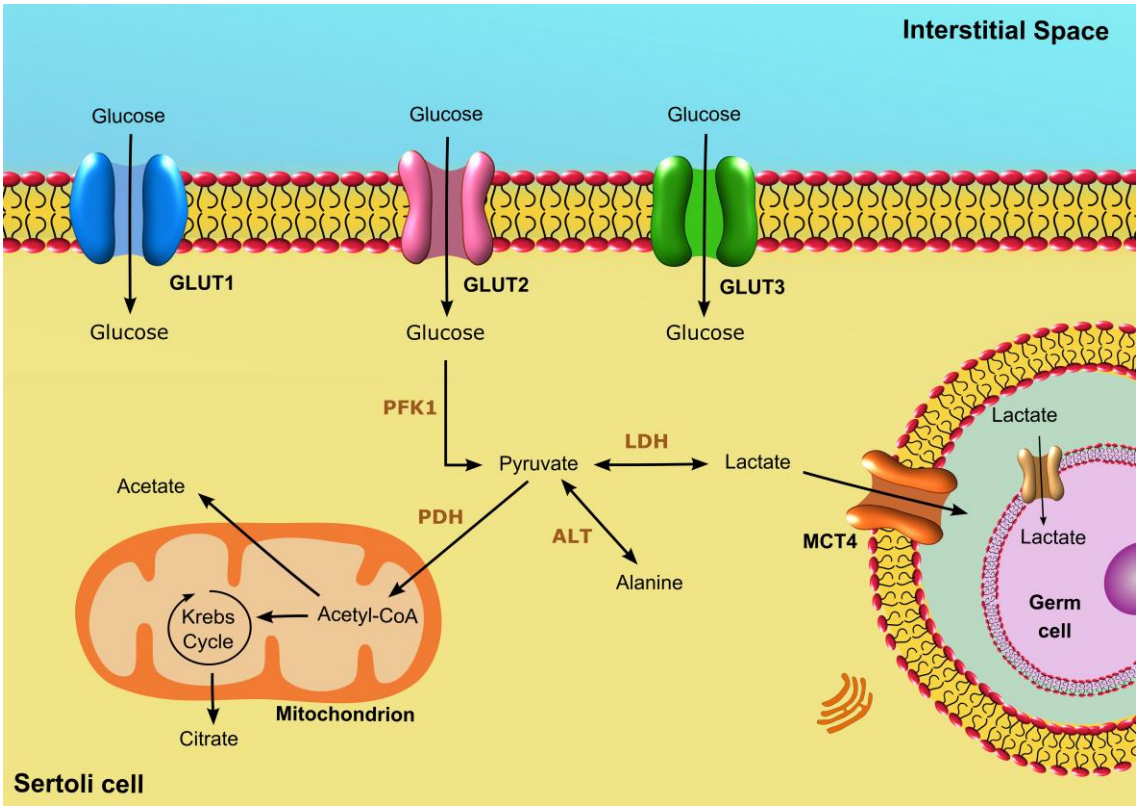

Figure 1.2 Sertoli cells (SCs) metabolic cooperation with developing germ cells. In SCs, glucose from the interstitial space is taken up through high-affinity glucose transporters, GLUT1, GLUT2 and GLUT3, which are present in the plasma membrane. In physiological conditions, most of glucose is converted to pyruvate through a rate-limiting process catalyzed by the enzyme phosphofructokinase 1 (PFK1). Pyruvate can be converted into lactate, alanine or acetyl-coA by the action of lactate dehydrogenase (LDH), alanine transaminase (ALT) or pyruvate dehydrogenase (PDH), respectively. Acetyl-CoA enters the mitochondrion to be used in the Krebs cycle, while lactate is exported to the intratubular fluid by specific monocarboxylate transporters (MCT4). This substrate is then taken up by germ cells to be used as metabolic fuel for adenosine triphosphate (ATP) production.

Spermatozoa ultrastructure

Spermatozoa present a unique and complex morphology. In general, they comprise a head, a midpiece and a tail region (Figure 1.3), commonly known as flagellum [15]. The spermatozoon is smaller than most cells in the body, but its size does not reflect its fundamental function of generating a new human being. The size, shape of the head, length and relative amount of the different components of the flagellum is species-specific [87]. For instance, rodent spermatozoa have hook-shaped heads, while the head of human spermatozoa is pear-shaped. The head of human spermatozoa has a median length of 4.4 µm and width of about 3 µm [88]. The nucleus occupies most of the human sperm head area and contains a haploid set of condensed, genetically inactive chromosomes [7]. The apical half of the nucleus is covered by the acrosome (Figure 1.3), which represents about 48% of the sperm head surface [88]. This

acrosomal cap is a membrane-enclosed cytoplasmic vesicle originating from the Golgi apparatus during spermatozoa formation [89]. It contains several monosaccharides (e.g. galactose, mannose, fructose) [90] and hydrolytic enzymes with a preponderant role in the penetration of the spermatozoon into the oocyte membranes [15]. The part of the nucleus that is not overlaid by the acrosome cap constitutes the postacrosomal region. The sperm head also contains a small amount of cytoplasm and several cytoskeletal structures, including the dense perinuclear layer that is made of basic proteins (e.g. calicin and cylicin I and II) associated with calmodulin and actin filaments [91]. On the base of the sperm head there is a small structure called connecting piece (or neck) that connects the head to the midpiece. The connecting piece harbors the proximal centriole and the empty vault. The proximal centriole is composed of nine microtubule triplets and has a vital role on the orchestration of cell division in the embryo, while the empty vault is originated after the degradation of the distal centriole during spermiogenesis [92]. The connecting piece provides a basal anchor to the axoneme and is covered by several redundant nuclear envelopes [93]. It was also proposed to be responsible for flagellum beat initiation and alternating directions of bends propagating down the beating flagellum [94].

The midpiece has a cylindrical shape and has about 5-9 μm of length and half the width of the sperm head (Figure 1.3) [95]. Its structure consists of numerous mitochondria spirally arranged around the outer dense fibers (ODF) and the central axial filament - axoneme [87]. The mitochondrial sheath is responsible for the production of ATP, which is the energy supply needed for tail motility during the migration upon the female reproductive tract. The axoneme is essentially a long, specialized cilium formed by a core of microtubules, surrounded by ODF extending from the connecting piece to the principal piece. It has a characteristic “9+2” structure, i.e., two central singlet microtubules encircled by nine outer doublet microtubules (A- and B-tubules). Radial links connect the central microtubule pair to each surrounding microtubule doublet, and nexin bridges connect adjacent doublets [96]. Outer and inner dyneins are observed as projecting “arms” that slide along each outer doublet microtubule. This active sliding has been associated with spermatozoa flagellar movement [97]. A cytoplasmic droplet is frequently found at the midpiece or at the junction of the midpiece with the principal piece in human mature spermatozoa. This tiny droplet-like structure is usually retained after the removal of spermatids cytoplasm by SCs at the end of spermiogenesis, and is suggested to play a key role in sperm volume adaptation [98]. At the distal part of the mitochondrial sheath of sperm midpiece is a traverse septin-based ring called annulus (Figure 1.3) that is the hallmark of the separation between the midpiece and the principal piece [99].

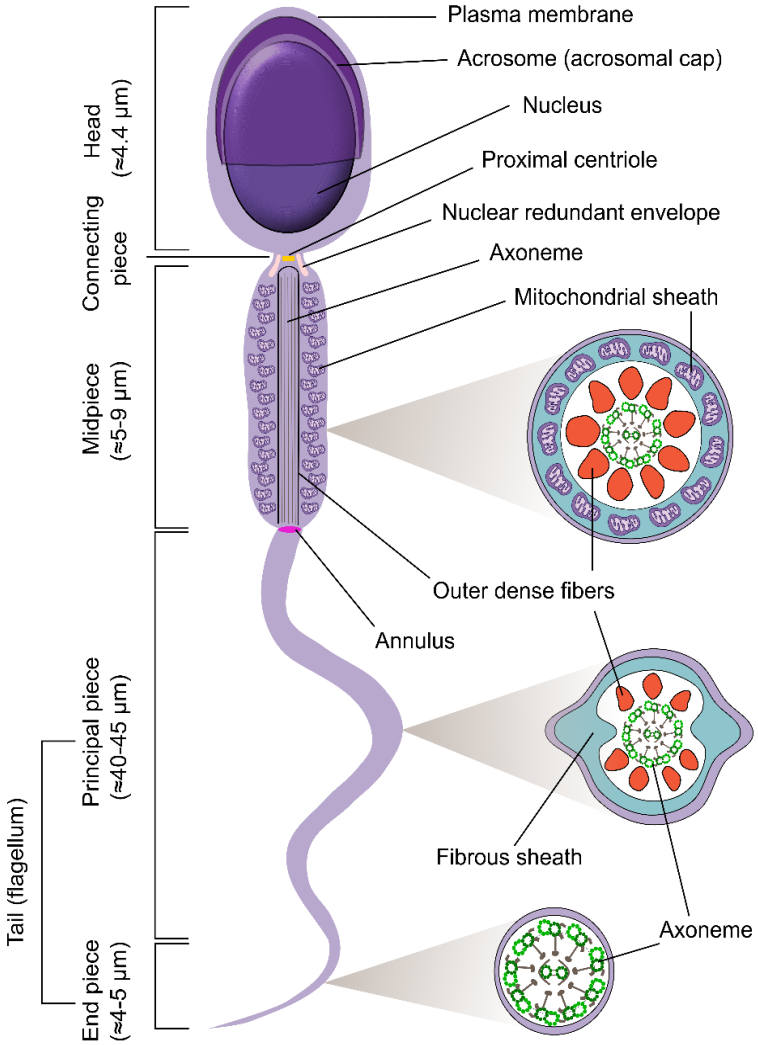

Figure 1.3 Human spermatozoon structure. It is constituted by the head, connecting piece, midpiece, principal piece and end piece. The head is mainly composed by the nucleus that is enveloped by the acrosome. The connecting piece contains the proximal centriole surrounded by the nuclear redundant envelopes. The midpiece holds the mitochondrial sheath, which is the site of energy production. These several mitochondria are organized around the outer dense fibers and the axoneme. The axoneme extends from the basis of the head (connecting piece) until the end of the sperm flagellum. Separating the midpiece and the beginning of the tail is a ring-like small structure called annulus. The tail comprises the principal piece that is the lengthiest part of the spermatozoon, and the end piece, the narrowest part. Besides the axoneme, the principal piece also contains the outer dense fibers surrounded by a fibrous sheath. The end piece is mainly composed by the axoneme. The spermatozoon is enveloped by a plasma membrane.

The sperm tail is the only functional flagellum in humans and it can be divided into principal piece and end piece. The principal piece constitutes most of the tail, having an average length of 40-45 µm [95]. It is constituted by the axoneme surrounded by a sheath of supportive fibers composed of two longitudinal columns that run parallel to ODF (Figure 1.3). The main function of principal piece is to propel the spermatozoon towards the oocyte, changing both the amplitude and frequency of the whip like movement of the tail to facilitate sperm hyperactivation and egg penetration.

Finally, the end piece is the narrowest part of the sperm and is about 4-5 μm long [95]. It consists of the axoneme and the ends of ODF and fibrous sheath [7]. Moreover, the whole spermatozoon is enveloped by a plasma membrane that presents a high lipid content, especially of polyunsaturated fatty acids (PUFAs) [100, 101]. This biochemical constitution confers a fluidity and flexibility to the sperm plasma membrane, which is essential for the fusion with the oocyte membrane at fertilization [101]. Overall, the morphological integrity of spermatozoa is vital for their adequate response to further maturational processes and for the acquisition of fertilizing ability.

Spermatozoa chromatin

The structural arrangement of sperm chromatin is vital for the proper functioning of these cells. The chromatin structure of spermatozoa is organized to resist to conditions that could damage the DNA. At the same time the chromatin must have the property of rapidly making the DNA available to the ooplasm. Sperm DNA is in the nucleus of the sperm head, occupying most of the nuclear volume. Though, it is the most tightly condensed eukaryotic DNA, being at least 6-fold more compacted than the DNA in somatic cells [102]. This is mostly due to the different types of DNA packaging. In the case of somatic cell nuclei and mitotic chromosomes, DNA is wrapped around histone octamers forming nucleosomes [103]. The nucleosomes are then further coiled into regular helixes called solenoids [104]. Concerning sperm DNA, during spermiogenesis, the vast majority of histones is replaced with transition proteins and then with protamines (Figure 1.4) [105]. During this process of remodeling/compaction of sperm chromatin, naturally occurring DNA strand breaks induced by topoisomerase II arise to relieve the torsional stresses that accompany the transition of sperm chromatin from an exclusively nucleosomal to a predominantly protamine-based configuration [106]. Protamines are small and highly basic proteins responsible for the higher degree of sperm chromatin compaction. Human sperm contain two types of protamines, P1 and P2, both present in roughly equal quantities [107]. Alteration in this 1:1 ratio have been correlated with general infertility and poor fertilization ability [108]. These protamines are very rich in positively charged arginine residues (55-79%), which neutralize the strong negative charges of the phosphate groups in the DNA backbone, thus permitting a strong DNA binding [109]. They also contain several cysteine residues that are responsible for conferring increased stability to sperm chromatin through multiple inter- and intraprotamine disulfide cross-links [109]. During the transit of spermatozoa through the epididymis, a final stage of chromatin organization occurs to originate an even more compacted chromatin. This process involves loss of water, an increase in the formation of disulfide bonds, and an additional reduction in the volume of sperm nuclei. Moreover, throughout this sperm epididymal maturation there is a complete restriction of the facilities for DNA replication and transcription [110]. Typically, this super-compacted sperm DNA occupies a 40-fold lower volume than somatic cells' DNA [111]. Furthermore, the human sperm

chromatin contains zinc, more specifically one zinc ion for each protamine molecule [107]. This feature allows the formation of zinc-stabilized structures (zinc fingers) among histidines and cysteines, thus locking the tertiary structure and thereby reducing the number of accessible conformations of the protamine. This increases the conformational stability of these proteins when interacting with DNA [105]. Protamines bounded to sperm DNA form doughnut-loop domains (Figure 1.4), known as toroids, that contain roughly 50 kb of DNA [112]. Protamine toroids may be organized to form a linear, side-by-side arrays of chromatin (Figure 1.4), resulting in an extremely condensed chromatin, in which most of DNA is hidden within the toroid [113]. Each protamine toroid contains a single DNA loop domain [114]. The toroid exists in a semi-crystalline state and is resistant to nuclease digestion, thus protecting DNA from degradation [115]. However, between each protamine toroid, there is a nuclease sensitive segment of chromatin called the toroid linker (Figure 1.4), which is also the site of attachment of DNA to sperm proteinaceous nuclear matrix, commonly known as matrix attachment regions (MARs) [116].

The protamine-based configuration of sperm DNA facilitates its protection, transport and safe delivery to the oocyte [117]. Nevertheless, the haploid chromatin of human sperm usually retains 5–15 % of histones [118], a higher percentage in comparison to other mammalian species (e.g., bulls, hamsters, and mice) that only retain up to 5 % [119]. Therefore, compared to other mammalian species, human sperm chromatin is relatively less compact. Histones are important for the regulation of the degree of DNA compaction and modulation of gene expression, since they can be modified by post-translational modifications (PTMs) that restrict or facilitate the access of transcription factors to the DNA [120]. Several histones have been identified in human sperm, including nuclear proteins histone 2A and 2B (H2A and H2B), histone 3 (H3), histone 4 (H4), and the testis-specific histone (tH2B) [121, 122]. Some studies have demonstrated that the distribution of histones throughout the sperm genome is not random, as they seem to be associated with specific genes [118, 123]. It has been suggested that histones are present in relatively large tracts of DNA, from 10 to 100 kb, and in smaller tracts of DNA interspersed throughout the genome. Moreover, the nuclease sensitivity at MARs suggests that these protamine linker regions are bounded by histones [118, 123].

After fertilization, sperm chromatin decondenses and protamines are completely replaced by histones in the first 2–4 h, so that the paternal chromatin has the same accessible chromatin as all other somatic cells [124]. Sperm nuclear matrix is also disrupted at fertilization, but sperm nuclei contain a unique structure called sperm nuclear annulus to which the entire complement of DNA seems to be anchored [125]. The sperm nuclear matrix acts as a checkpoint for sperm DNA integrity after fertilization, being essential for DNA replication [112]. While protamines are unique to mature spermatozoa, histones and MARs are both found in somatic cells and can be presumed to be residual from the sperm progenitor cells from which spermatozoa are produced [112]. These findings provide evidence for the protective role of protamines during the transit of spermatozoa from testis to the moment of fertilization. The structural

organization of both histone-bound chromatin and sperm MARs are probably transmitted to the newly formed paternal pronucleus after fertilization and suggests that both are required for proper embryogenesis. It is possible that the newly fertilized oocytes inherit histone-based chromatin structural organization from the sperm (Figure 1.4) [112]. These intricate features of sperm chromatin are essential for sperm function and for the success of fertilization.

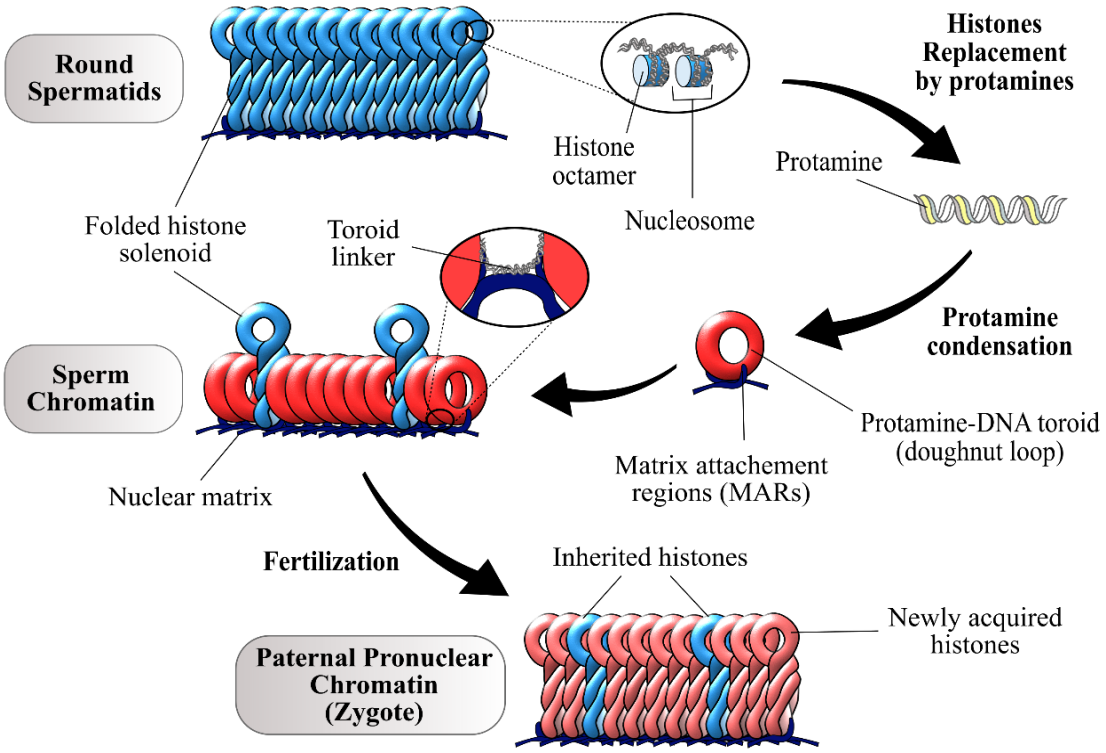

Figure 1.4 Human sperm chromatin remodelling. The DNA of round spermatids is typically bounded to histone octamers, forming nucleosomes. These structures are folded into regular loops called solenoids. During spermatogenesis, most of this histone-based chromatin is replaced by protamines. However, 5-15% of the histones are retained. Protamines condensation leads to the formation of doughnut loops, known as toroids, to increase DNA protection. Protamine toroids are usually organized side-by-side, linked between each other by a nuclease sensitive segment of chromatin called the toroid linker. This structure also constitutes the site of attachment of DNA to the nuclear matrix - matrix attachment regions (MARs). At fertilization, the sperm chromatin decondenses and protamines are completely replaced by histones. The histones that have not been replaced during spermatogenesis are the inherited ones, whereas those that have been replaced are, at this point, newly acquired histones. It is believed that the retained histones carry the paternal epigenetic marks to the zygote. The MARs are also probably retained in the paternal pronucleus.

Spermatozoa maturation

Spermatozoa development goes far beyond their complex process of production in the seminiferous tubules. Their post-testicular progress involves ultrastructural and macromolecular modifications during their course to reach the site of fertilization in the oviduct [126]. These alterations result from sequential, temporally controlled interactions between male reproductive tract secretions and the transiting male gamete [127]. After being shed from the testes, spermatozoa follow their pathway through the rete testis and efferent

ducts, reaching the epididymis. The epididymis is a long convoluted tubule that is structurally organized into three principal regions: the caput (head), the corpus (body) and the distal cauda epididymis (tail) (Figure 1.5) [128]. The epididymal epithelium is constituted by several cell types: principal, narrow, apical, clear, basal, halo and surrounding peritubular myoid cells [44, 129]. Each cell type has a typical function and compartmentalization throughout the epididymal duct (Figure 1.5). Though, all cellular types contribute to the establishment and regulation of the epididymal luminal environment, which is crucial for the attainment of spermatozoa peculiar morphological and functional characteristics [126, 129]. Among the wide range of epididymal functions, we can highlight the following: (1) sperm concentration, to facilitate ejaculation; (2) functional maturation, to acquire motility and fertilizing ability; (3) storage in a quiescent viable state until ejaculation; (4) removal of degenerating sperm and (5) protection of spermatozoa [130].

From the rete testis to the end of the epididymis, spermatozoa are bathed in a continuously and progressively changing medium of fluid proteins and other chemical components [131]. The epididymal fluid composition is highly regulated by a selective structure known as blood-epididymal barrier (BEB). The formation of this barrier is a critical factor for sperm maturation, as it enables the development of the proper epididymal luminal environment for spermatozoa maturational process. Besides, the epididymis has a great proteinaceous secretory activity [132]. In fact, it releases a wide range of proteins that directly influence the composition of the epididymal epithelium (e.g. pH, osmolality), but also contributes for sperm protection through the modulation of oxidative stress (OS) [133]. The maturational process of spermatozoa includes many changes in sperm physiological properties, such as the acquisition of forward motility, the ability to recognize and bind to the zona pellucida, and the capacity to fuse with the plasma membrane of an oocyte [134]. During epididymal transit, spermatozoa acquire new proteins, some of which are coating proteins that can be removed by washing with isotonic or hypertonic solutions, while others are assimilated by sperm plasma membrane as integral membrane proteins. These latter are incorporated in spermatozoa membranes by small vesicles called epididymosomes [135]. However, the underlying mechanisms are not yet fully understood.

The mature spermatozoa are finally stored in a quiescent state in the cauda epididymis [136]. As they can be stored for several days until ejaculation, the characteristic microenvironment of this epididymal section should also be controlled. Particularly, there is a need to protect spermatozoa because they are highly susceptible to damage by high levels of reactive oxygen species (ROS) [101]. Moreover, at this stage, it is essential to remove degenerating spermatozoa, as they can damage the viable quiescent cells [137]. The biochemical sequential modifications of spermatozoa during their transit through the epididymis, transform the immotile testicular spermatozoa into the quiescent, but actively motile, spermatozoa at the end of the epididymal duct.

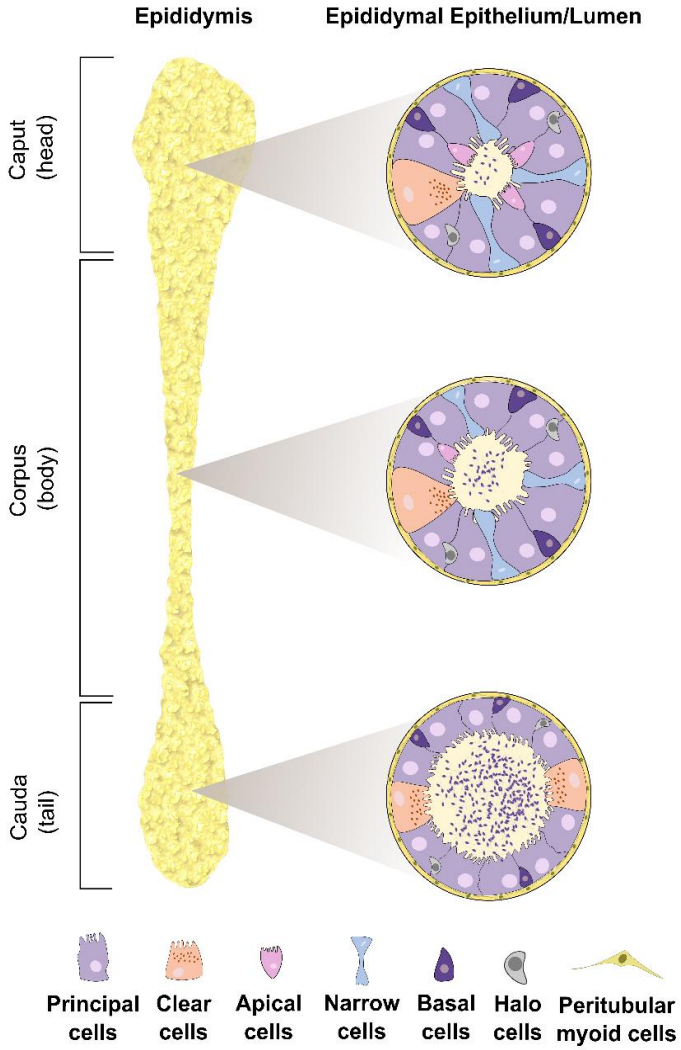

Figure 1.5 Human epididymal duct. It is constituted by the caput (head), corpus (body) and cauda (tail). The epididymal epithelium comprises a diverse set of cell types: principal, clear, apical, narrow, basal, halo and peritubular myoid cells. There are evident differences in the distribution of epithelial cells along each epididymal segment. Principal cells are the most abundant cells, being largely present in caput region and gradually decreasing until cauda region. Narrow and apical cells are only present in caput and corpus regions, and their expression decreases from the proximal to the distal segment. Clear and basal cells are present in all epididymal regions, though the percentage of basal cells is higher. Besides, clear cells are more active in the cauda epididymis. Halo cells are small cells located on the base of all the epithelium, but do not touch the basement membrane. Peritubular myoid cells are the cells that surround all the other cellular types. The diameter of the epididymal lumen, as well as the number of spermatozoa, increases along the epididymal duct.

During ejaculation, spermatozoa are subjected to changes in the external medium composition, passing through high ionic strength fluids of low osmolality, low K^+ and high Na^+, which contribute for motility activation [138]. The flagellum of an activated sperm generates a symmetrical, lower-amplitude waveform that drives the sperm almost in straight line in relatively non-viscous media, such as seminal plasma [139]. In addition, mammalian sperm display other type of physiological motility, the so-called hyperactivated motility, as seen in most sperm collected from the fertilization site. At some point after the spermatozoon reaches the oviduct, the flagellum is hyperactivated. Subsequently, the pattern of the flagellar beat changes to an asymmetric movement of higher amplitude, resulting in sudden changes in the

direction of travel [140, 141]. It has been suggested that hyperactivated motility helps sperm to detach from the oviductal epithelium, move progressively through the oviductal environment to reach the site of fertilization, and penetrate the egg membrane [142].

References

1. Sikka SC and Wang R (2008) Endocrine disruptors and estrogenic effects on male reproductive axis. Asian journal of andrology 10(1):134-45.
2. Su L, et al. (2011) Drug transporters, the blood-testis barrier, and spermatogenesis. The Journal of Endocrinology 208(3):207-23.
3. Wang R-S, et al. (2009) Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. Endocrine reviews 30(2):119-32.
4. Bettegowda A and Wilkinson MF (2010) Transcription and post-transcriptional regulation of spermatogenesis. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 365(1546):1637-51.
5. Han X, et al. (2009) Transplantation of sertoli-islet cell aggregates formed by microgravity: prolonged survival in diabetic rats. Experimental Biology and Medicine 234(5):595-603.
6. Cheng C, et al. (2010) Regulation of spermatogenesis in the microenvironment of the seminiferous epithelium: new insights and advances. Molecular and Cellular Endocrinology 315(1-2):49-56.
7. Saladin KS (2003) The male reproductive system. Anatomy and Physiology: The unit of form and function, McGraw−Hill, pp. 1017-47
8. Kidd SA, et al. (2001) Effects of male age on semen quality and fertility: a review of the literature. Fertility and sterility 75(2):237-48.
9. Papaioannou M and Nef S (2010) microRNAs in the testis: building up male fertility. Journal of Andrology 31(1):26-33.
10. O'Donnell L, et al. (2011) Spermiation: The process of sperm release. Spermatogenesis 1(1):14-35.
11. França LR, et al. (1998) Germ cell genotype controls cell cycle during spermatogenesis in the rat. Biology of reproduction 59(6):1371-77.
12. Hess RA and de Franca LR (2009) Spermatogenesis and cycle of the seminiferous epithelium. Molecular mechanisms in spermatogenesis, Springer, pp. 1-15
13. Aragon M, et al. (2005) Serotoninergic system blockage in the prepubertal rat inhibits spermatogenesis development. Reproduction 129(6):717-27.
14. Walker WH and Cheng J (2005) FSH and testosterone signaling in Sertoli cells. Reproduction 130(1):15-28.
15. Vander AJ, et al. (2001) Human Physiology: The Mechanism of Body Function. McGraw Hill.
16. Kekenes-Huskey PM, et al. (2004) A molecular docking study of estrogenically active compounds with 1, 2-diarylethane and 1, 2-diarylethene pharmacophores. Bioorganic & medicinal chemistry 12(24):6527-37.
17. Mruk DD and Cheng CY (2011) Desmosomes in the testis: Moving into an unchartered territory. Spermatogenesis 1(1):47-51.
18. Jégou B (1992) The Sertoli cell in vivo and in vitro. Cell biology and toxicology 8(3):49-54.
19. Mackay S (2000) Gonadal development in mammals at the cellular and molecular levels. International review of cytology 200:47-99.
20. Hacker A, et al. (1995) Expression of Sry, the mouse sex determining gene. Development 121(6):1603-14.
21. Chaudhary J, et al. (2005) The helix-loop-helix inhibitor of differentiation (ID) proteins induce post-mitotic terminally differentiated Sertoli cells to re-enter the cell cycle and proliferate. Biology of Reproduction 72(5):1205-17.
22. Ryser S, et al. (2011) Gene expression profiling of rat spermatogonia and Sertoli cells reveals signaling pathways from stem cells to niche and testicular cancer cells to surrounding stroma. BMC genomics 12(1):29.
23. Tran D and Josso N (1982) Localization of anti-Müllerian hormone in the rough endoplasmic reticulum of the developing bovine Sertoli cell using immunocytochemistry with a monoclonal antibody. Endocrinology 111(5):1562-67.
24. De Santa Barbara P, et al. (1998) Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Müllerian hormone gene. Molecular and cellular biology 18(11):6653-65.
25. Sharpe RM, et al. (2003) Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction 125(6):769-84.

26. De Kretser D and Kerr J (1988) The cytology of the testis. In: E Knobil and J Neill (eds) The physiology of reproduction, Raven Press, New York pp. 837-932
27. Bremner WJ, et al. (1994) Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. Endocrinology 135(3):1227-34.
28. Ketola I, et al. (2002) Developmental expression and spermatogenic stage specificity of transcription factors GATA-1 and GATA-4 and their cofactors FOG-1 and FOG-2 in the mouse testis. European journal of endocrinology 147(3):397-406.
29. Holsberger DR, et al. (2003) Thyroid hormone regulates the cell cycle inhibitor p27Kip1 in postnatal murine Sertoli cells. Endocrinology 144(9):3732-38.
30. Law GL and Griswold MD (1994) Activity and form of sulfated glycoprotein 2 (clusterin) from cultured Sertoli cells, testis, and epididymis of the rat. Biology of Reproduction 50(3):669-79.
31. Schlatt S, et al. (1996) Discriminative analysis of rat Sertoli and peritubular cells and their proliferation in vitro: evidence for follicle-stimulating hormone-mediated contact inhibition of Sertoli cell mitosis. Biology of Reproduction 55(2):227-35.
32. Norton JN and Skinner MK (1989) Regulation of Sertoli cell function and differentiation through the actions of a testicular paracrine factor P-Mod-S. Endocrinology 124(6):2711-19.
33. Shinohara T, et al. (2003) Restoration of spermatogenesis in infertile mice by Sertoli cell transplantation. Biology of reproduction 68(3):1064-71.
34. Zhang Z, et al. (2008) Bovine sertoli cells colonize and form tubules in murine hosts following transplantation and grafting procedures. Journal of andrology 29(4):418-30.
35. Oliveira PF, et al. (2009) Intracellular pH regulation in human Sertoli cells: role of membrane transporters. Reproduction 137(2):353-59.
36. Chui K, et al. (2011) Characterization and functionality of proliferative human Sertoli cells. Cell transplantation 20(5):619-35.
37. Reis MM, et al. (2015) Sertoli cell as a model in male reproductive toxicology: advantages and disadvantages. Journal of Applied Toxicology 35(8):870-83.
38. Wong C-H and Cheng CY (2005) The Blood-Testis Barrier: Its Biology, Regulation, and Physiological Role in Spermatogenesis. Current topics in developmental biology 71:263-96.
39. Cheng CY and Mruk DD (2009) An intracellular trafficking pathway in the seminiferous epithelium regulating spermatogenesis: a biochemical and molecular perspective. Critical reviews in biochemistry and molecular biology 44(5):245-63.
40. Cheng CY and Mruk DD (2010) A local autocrine axis in the testes that regulates spermatogenesis. Nature Reviews Endocrinology 6(7):380-95.
41. Li MW, et al. (2009) Cytokines and junction restructuring events during spermatogenesis in the testis: an emerging concept of regulation. Cytokine & growth factor reviews 20(4):329-38.
42. Cheng CY, et al. (2010) Regulation of spermatogenesis in the microenvironment of the seminiferous epithelium: new insights and advances. Molecular and cellular endocrinology 315(1):49-56.
43. Mruk DD and Cheng CY (2004) Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. Endocrine reviews 25(5):747-806.
44. Mital P, et al. (2011) The blood-testis and blood-epididymis barriers are more than just their tight junctions. Biology of Reproduction 84(5):851-58.
45. Rato L, et al. (2010) Tubular fluid secretion in the seminiferous epithelium: ion transporters and aquaporins in Sertoli cells. The Journal of Membrane Biology 236(2):215-24.
46. Oliveira PF, et al. (2009) Membrane transporters and cytoplasmatic pH regulation on bovine Sertoli cells. The Journal of Membrane Biology 227(1):49-55.
47. Toyama Y, et al. (2003) Ectoplasmic specializations in the Sertoli cell: new vistas based on genetic defects and testicular toxicology. Anatomical science international 78(1):1-16.
48. Rato L, et al. (2012) Metabolic regulation is important for spermatogenesis. Nature Reviews Urology 9(6):330-38.
49. Griswold MD (1995) Interactions between germ cells and Sertoli cells in the testis. Biology of Reproduction 52(2):211-16.
50. Weber JE, et al. (1983) Three-dimensional reconstruction of a rat stage V Sertoli cell: II. Morphometry of Sertoli--Sertoli and Sertoli--germ-cell relationships. The American Journal of Anatomy 167(2):163-79.
51. Tripathi R, et al. (2009) Male germ cell development: turning on the apoptotic pathways. Journal of Reproductive Immunology 83(1-2):31-5.
52. Orth JM, et al. (1988) Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. Endocrinology 122(3):787-94.
53. Chung SS, et al. (1999) Study on the formation of specialized inter-Sertoli cell junctions in vitro. Journal of cellular physiology 181(2):258-72.
54. Sertoli E (1865) Dell'esistenza di particolari cellule ramificate nei canalicoli seminiferi del testicolo umano. Morgagni 7:31-40.

55. Vogl AW, et al. (2000) Unique and Multifunctional Adhesion Junctions in the Testis. Ectoplasmic Specializations. Archives of histology and cytology 63(1):1-15.
56. Russell L (1993) Morphological and functional evidence for Sertoli-germ cell relationships. In: LD Russell and MD Griswold (eds) The Sertoli Cell, Cache River Press, Clearwater, FL, USA, pp. 365-90.
57. Griswold MD (1988) Protein secretions of Sertoli cells. International Review of Cytology 110:133-56.
58. Skinner M (1993) Secretion of growth factors and other regulatory factors. In: LD Russell and MD Griswold (eds) The Sertoli Cell, Cache River Press, Clearwater, FL, USA, pp. 237-48.
59. Sylvester S (1993) Secretion of transport and binding proteins. In: LD Russell and MD Griswold (eds) The Sertoli Cell, Cache River Press, Clearwater, FL, USA, pp. 201-16.
60. Skinner MK and Griswold MD (2005) Sertoli cell secreted regulatory factors. Sertoli cell biology, Elsevier academic press, San Diego pp. 107–20.
61. Galardo MN, et al. (2008) Regulation of expression of Sertoli cell glucose transporters 1 and 3 by FSH, IL1 beta, and bFGF at two different time-points in pubertal development. Cell and Tissue Research 334(2):295-304.
62. Robinson R and Fritz IB (1981) Metabolism of glucose by Sertoli cells in culture. Biology of Reproduction 24(5):1032-41.
63. Oliveira PF, et al. (2012) Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells. Biochimica et Biophysica Acta 1820(2):84-89.
64. Martins AD, et al. (2013) Control of Sertoli cell metabolism by sex steroid hormones is mediated through modulation in glycolysis-related transporters and enzymes. Cell and Tissue Research 354(3):861-68.
65. Grootegoed JA, et al. (1986) Metabolism of radiolabelled energy-yielding substrates by rat Sertoli cells. Journal of Reproduction and Fertility 77(1):109-18.
66. Mann T (1949) Metabolism of semen. In: FF Nord (ed) Advances in Enzymology and Related Areas of Molecular Biology, vol. 9, Wiley, Hoboken, NJ, USA, pp. 234-329.
67. Rato L, et al. (2012) Metabolic modulation induced by oestradiol and DHT in immature rat Sertoli cells cultured in vitro. Bioscience Reports 32(1):61-9.
68. Alves MG, et al. (2012) In vitro cultured human Sertoli cells secrete high amounts of acetate that is stimulated by 17beta-estradiol and suppressed by insulin deprivation. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1823(8):1389-94.
69. Erkkila K, et al. (2002) Lactate inhibits germ cell apoptosis in the human testis. Molecular Human Reproduction 8(2):109-17.
70. Riera M, et al. (2002) Regulation of lactate production and glucose transport as well as of glucose transporter 1 and lactate dehydrogenase A mRNA levels by basic fibroblast growth factor in rat Sertoli cells. Journal of Endocrinology 173(2):335-43.
71. Oliveira PF, et al. (2011) Influence of 5alpha-dihydrotestosterone and 17beta-estradiol on human Sertoli cells metabolism. International Journal of Andrology 34(6 Pt 2):e612-20.
72. Grootegoed JA, et al. (1984) The role of glucose, pyruvate and lactate in ATP production by rat spermatocytes and spermatids. Biochimica et Biophysica Acta 767(2):248-56.
73. Riera MF, et al. (2009) Molecular mechanisms involved in Sertoli cell adaptation to glucose deprivation. American Journal of Physiology, Endocrinology and Metabolism 297(4):E907-14.
74. Courtens JL and Ploen L (1999) Improvement of spermatogenesis in adult cryptorchid rat testis by intratesticular infusion of lactate. Biology of Reproduction 61(1):154-61.
75. Nakamura M, et al. (1984) Metabolism of pachytene primary spermatocytes from rat testes: pyruvate maintenance of adenosine triphosphate level. Biology of Reproduction 30(5):1187-97.
76. Jutte NH, et al. (1981) Exogenous lactate is essential for metabolic activities in isolated rat spermatocytes and spermatids. Journal of Reproduction and Fertility 62(2):399-405.
77. Mita M and Hall PF (1982) Metabolism of round spermatids from rats: lactate as the preferred substrate. Biology of Reproduction 26(3):445-55.
78. Mann T (1964) Metabolism of semen: fructolysis, respiration and sperm energetics. The biochemistry of semen and of the male reproductive tract, Methuen, London pp. 265-307
79. Boussouar F and Benahmed M (2004) Lactate and energy metabolism in male germ cells. Trends in Endocrinology and Metabolism 15(7):345-50.
80. Miki K (2007) Energy metabolism and sperm function. Society of Reproduction and Fertility Supplement 65:309-25.
81. Miyashita Y, et al. (2007) Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in Arabidopsis thaliana. The Plant Journal 49(6):1108-21.
82. Dias TR, et al. (2014) Sperm glucose transport and metabolism in diabetic individuals. Molecular and cellular endocrinology 396(1):37-45.
83. Alves MG, et al. (2013) Molecular mechanisms beyond glucose transport in diabetes-related male infertility. Biochimica et Biophysica Acta 1832(5):626-35.
84. Costello LC and Franklin RB (2006) The clinical relevance of the metabolism of prostate cancer; zinc and tumor suppression: connecting the dots. Molecular cancer 5(1):17.

85. Yamashita H, et al. (2006) Acetate generation in rat liver mitochondria; acetyl-CoA hydrolase activity is demonstrated by 3-ketoacyl-CoA thiolase. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 1761(1):17-23.
86. Shimazu T, et al. (2010) Acetate metabolism and aging: an emerging connection. Mechanisms of ageing and development 131(7):511-16.
87. Rabbani SI, et al. (2010) Pioglitazone, a PPAR-gamma ligand inhibited the nicotinamide-streptozotocin induced sperm abnormalities in type-2 diabetic Wistar rats. Pakistan Journal of Pharmaceutical Sciences 23(3):326-31.
88. Soler C and Sinisi AA (2010) Dimensions of human ejaculated spermatozoa in Papanicolaou-stained seminal and swim-up smears obtained from the Integrated Semen Analysis System (ISAS®). Asian journal of andrology 12:871-79.
89. Yao R, et al. (2002) Lack of acrosome formation in mice lacking a Golgi protein, GOPC. Proceedings of the National Academy of Sciences 99(17):11211-16.
90. Boström K and Öckerman P (1971) Glycosidases in human semen and male genital organs. Scandinavian journal of urology and nephrology 5(2):117-22.
91. Virtanen I, et al. (1984) Distinct cytoskeletal domains revealed in sperm cells. The Journal of Cell Biology 99(3):1083-91.
92. Sathananthan AH, et al. (2000) Characterization of human gamete centrosomes for assisted reproduction. Italian Journal of Anatomy and Embryology 106(2 Suppl 2):61-73.
93. De Jonge C and Barratt C (2006) The sperm cell: production, maturation, fertilization, regeneration. Cambridge University Press, United Kingdom.
94. Vernon GG and Woolley DM (2004) Basal sliding and the mechanics of oscillation in a mammalian sperm flagellum. Biophysical journal 87(6):3934-44.
95. Saladin KS (2007) Human Anatomy. McGraw–Hill, 7th edition.
96. Gaffney EA, et al. (2011) Mammalian sperm motility: observation and theory. Annual Review of Fluid Mechanics 43:501-28.
97. Inaba K (2011) Sperm flagella: comparative and phylogenetic perspectives of protein components. Molecular human reproduction 17(8):524-38.
98. Xu H, et al. (2013) The cytoplasmic droplet may be indicative of sperm motility and normal spermiogenesis. Asian journal of andrology 15(6):799-805.
99. Lhuillier P, et al. (2009) Absence of annulus in human asthenozoospermia: Case Report. Human reproduction 24(6):1296-303.
100. Flesch FM and Gadella BM (2000) Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes 1469(3):197-235.
101. Lenzi A, et al. (2000) Lipoperoxidation damage of spermatozoa polyunsaturated fatty acids (PUFA): scavenger mechanisms and possible scavenger therapies. Frontiers in Bioscience 5(1):1-15.
102. Ward WS and Zalensky AO (1996) The unique, complex organization of the transcriptionally silent sperm chromatin. Critical Reviews in Eukaryotic Gene Expression 6(2-3):139-47.
103. Ioannou D, et al. (2016) Impact of sperm DNA chromatin in the clinic. Journal of assisted reproduction and genetics 33(2):157-66.
104. Finch JT and Klug A (1976) Solenoidal model for superstructure in chromatin. Proceedings of the National Academy of Sciences 73(6):1897-901.
105. Björndahl L and Kvist U (2010) Human sperm chromatin stabilization: a proposed model including zinc bridges. Molecular human reproduction 16(1):23-29.
106. Tarozzi N, et al. (2007) Clinical relevance of sperm DNA damage in assisted reproduction. Reproductive biomedicine online 14(6):746-57.
107. Balhorn R (2007) The protamine family of sperm nuclear proteins. Genome biology 8(9):1.
108. Zhang X, et al. (2006) Sperm nuclear histone to protamine ratio in fertile and infertile men: evidence of heterogeneous subpopulations of spermatozoa in the ejaculate. Journal of andrology 27(3):414-20.
109. Agarwal A and Said TM (2003) Role of sperm chromatin abnormalities and DNA damage in male infertility. Human Reproduction Update 9(4):331-45.
110. Jennings C and Powell D (1995) Genome organisation in the murine sperm nucleus. Zygote 3(2):123-31.
111. Ward WS (1994) The structure of the sleeping genome: implications of sperm DNA organization for somatic cells. Journal of cellular biochemistry 55(1):77-82.
112. Ward WS (2010) Function of sperm chromatin structural elements in fertilization and development. Molecular human reproduction 16(1):30-36.
113. Miller D, et al. (2010) Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. Reproduction 139(2):287-301.
114. Sotolongo B, et al. (2005) An Endogenous Nuclease in Hamster, Mouse, and Human Spermatozoa Cleaves DNA into Loop-Sized Fragments. Journal of andrology 26(2):272-80.
115. Ward MA and Ward WS (2004) A model for the function of sperm DNA degradation. Reproduction, Fertility and Development 16(5):547-54.
116. Martins RP, et al. (2004) Nuclear matrix interactions at the human protamine domain: a working model of potentiation. Journal of Biological Chemistry 279(50):51862-68.

117. Rousseaux S, et al. (2005) Establishment of male-specific epigenetic information. Gene 345(2):139-53.
118. Hammoud SS, et al. (2009) Distinctive chromatin in human sperm packages genes for embryo development. Nature 460(7254):473-78.
119. Erenpreiss J, et al. (2006) Sperm chromatin structure and male fertility: biological and clinical aspects. Asian journal of andrology 8(1):11-29.
120. Bowman GD and Poirier MG (2014) Post-translational modifications of histones that influence nucleosome dynamics. Chemical reviews 115(6):2274-95.
121. Gatewood JM, et al. (1990) Isolation of four core histones from human sperm chromatin representing a minor subset of somatic histones. Journal of Biological Chemistry 265(33):20662-66.
122. Zalensky AO, et al. (1993) Organization of centromeres in the decondensed nuclei of mature human sperm. Chromosoma 102(8):509-18.
123. Arpanahi A, et al. (2009) Endonuclease-sensitive regions of human spermatozoal chromatin are highly enriched in promoter and CTCF binding sequences. Genome research 19(8):1338-49.
124. Ajduk A, et al. (2006) Sperm chromatin remodeling after intracytoplasmic sperm injection differs from that of in vitro fertilization. Biology of reproduction 75(3):442-51.
125. Barone JG, et al. (1994) DNA organization in human spermatozoa. Journal of andrology 15:139-39.
126. Cornwall GA (2009) New insights into epididymal biology and function. Human Reproduction Update 15(2):213-27.
127. Toshimori K (2003) Biology of spermatozoa maturation: an overview with an introduction to this issue. Microscopy research and technique 61(1):1-6.
128. Kent M (2001) Male Reproductive System. Human anatomy, McGraw-Hill, pp. 697-724
129. Shum WW, et al. (2011) Establishment of cell-cell cross talk in the epididymis: control of luminal acidification. Journal of Andrology 32(6):576-86.
130. Marengo SR (2008) Maturing the sperm: unique mechanisms for modifying integral proteins in the sperm plasma membrane. Animal reproduction science 105(1):52-63.
131. Guyonnet B, et al. (2011) The epididymal transcriptome and proteome provide some insights into new epididymal regulations. Journal of Andrology 32(6):651-64.
132. Dacheux JL, et al. (2003) Contribution of epididymal secretory proteins for spermatozoa maturation. Microscopy Research and Technique 61(1):7-17.
133. Gatti JL, et al. (2004) Post-testicular sperm environment and fertility. Animal reproduction science 82-83:321-39.
134. Sullivan R and Mieusset R (2016) The human epididymis: its function in sperm maturation. Human Reproduction Update 22(5):574-87.
135. Sullivan R, et al. (2007) Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. Asian Journal of Andrology 9(4):483-91.
136. Foldesy RG and Bedford JM (1982) Biology of the scrotum. I. Temperature and androgen as determinants of the sperm storage capacity of the rat cauda epididymidis. Biology of reproduction 26(4):673-82.
137. Jones R (2004) Sperm survival versus degradation in the mammalian epididymis: a hypothesis. Biology of Reproduction 71(5):1405-11.
138. Cooper TG (2011) The epididymis, cytoplasmic droplets and male fertility. Asian journal of andrology 13(1):130-38.
139. Turner RM (2005) Moving to the beat: a review of mammalian sperm motility regulation. Reproduction, Fertility and Development 18(2):25-38.
140. Suarez SS and Ho HC (2003) Hyperactivated motility in sperm. Reproduction in Domestic Animals 38(2):119-24.
141. Suarez SS (2008) Control of hyperactivation in sperm. Human reproduction update 14(6):647-57.
142. Ho H-C and Suarez SS (2001) Hyperactivation of mammalian spermatozoa: function and regulation. Reproduction 122(4):519-26.

Diabetes *mellitus* and male fertility

This section was adapted from the following publications:

- **Dias TR**, Alves MG, Neuhaus-Oliveira A, Socorro S, Silva BM, Oliveira PF. (2013) "Implications of Diabetes on sperm glucose uptake and metabolism" In Glucose Uptake: Regulation, Signaling Pathways and Health Implications, Nova Science Publishers, Inc, New York, USA, pp. 141-168 (ISBN: 978-1-62618-671-2).

- **Dias TR**, Alves MG, Silva BM, Oliveira PF. (2014) "Sperm glucose transport and metabolism in diabetic individuals". Molecular and Cellular Endocrinology 396(1-2):37-45 (DOI:10.1016/j.mce.2014.08.005).

- **Dias TR**, Bernardino RL, Meneses MJ, Sousa M, Sá R, Alves MG, Silva BM, Oliveira PF. (2016) "Emerging potential of natural products as an alternative strategy to pharmacological agents used against metabolic disorders". Current Drug Metabolism 17(6):582-597 (DOI:10.2174/1389200217666160229113629).

- **Dias TR**, Alves MG, Casal S, Oliveira PF, Silva BM. (2017) "Promising potential of dietary (poly)phenolic compounds in the prevention and treatment of diabetes *mellitus*" Current Medicinal Chemistry 24(4):334-354 (DOI:10.2174/0929867323666160905150419).

Diabetes *mellitus*

Proteins, carbohydrates, and fats are broken down by enzymes in the human digestive system, and then carried to the cells where they can be used as fuel. The body either uses these substances immediately, or stores them in the liver, body fat, and muscle tissues for later use. The dysregulation of these processes constitutes the onset of metabolic disorders, causing the production of either too much or too little of the body's essential substances. Many human diseases are caused by, or result in, an abnormal metabolic state, being diabetes *mellitus* (DM) one of the most worrying. Over the last few decades, DM has evolved into a global epidemic. According to the World Health Organization (WHO), in 2016, DM was the direct cause of 1.6 million deaths, being considered the 7th leading cause of death worldwide [1]. Besides, high blood glucose was responsible for 2.2 million deaths [1].

DM is a treatable but incurable lifelong disease characterized by a hyperglycemic state. Individuals with this condition present defects in protein, fat and carbohydrate metabolism, mostly due to a malfunctioning in insulin secretion, insulin action, or both [2, 3], defects in ROS production and scavenging defenses [4], and high OS [5, 6]. The number of diabetic individuals has been rapidly increasing worldwide. In 2016, 422 million people were diagnosed with DM [1], and this number is estimated to reach 592 million people in 2035, thus affecting more than one in 10 adults worldwide [7]. The increasing prevalence of obesity, along with the lack of physical activity, the growth of global population, aging and urbanization are strong contributors to the epidemic proportions of DM [8]. There are two main types of DM: type 1 (T1DM) and type 2 (T2DM). T1DM accounts for 5-10% of the total DM cases. Most of T1DM cases are caused by an autoimmune reaction in which the body's defense system attacks insulin-producing pancreatic beta cells in genetically susceptible individuals, culminating in their destruction (type 1a) [9, 10]. However, a small minority of cases result from an idiopathic destruction or failure of beta cells (type 1b). T1DM develops more often in children and young adults, but it may affect people at any age [11]. Individuals with T1DM produce very little or no insulin, so, to survive, they need to control their blood glucose levels with exogenous insulin. The pathogenic factors that lead to T1DM are not fully elucidated yet. However, there is clear evidence that it develops due to alterations in the immune regulation [12]. On the other hand, T2DM is responsible for 90-95% of all DM cases [3], and is characterized by insulin resistance (IR), which is described as the inability of cells to respond to normal circulating levels of insulin [13]. Despite the genetic predisposition, the risk of developing T2DM in humans increases with age, obesity, cardiovascular diseases and lack of physical activity [14, 15]. Generally, these individuals do not need exogenous treatment with insulin to survive and for that reason T2DM is commonly known as non-insulin-dependent DM [3]. The development of T2DM is a progressive process due to the limited capacity of pancreatic cells to augment the secretion of insulin to counterbalance IR, maintaining glucose tolerance at normal levels. However, eventually, this compensation is committed and impaired glucose tolerance (IGT) develops [16], originating the

so-called prediabetes, an intermediate state between normal glucose tolerance and evident T2DM [17]. In 2017, it was estimated that prediabetes affected about 34% of the adult US population [18] and it is expected to affect 107.7 million people in 2030 [19]. Prediabetes is a warning status for the predisposition of developing T2DM [20, 21]. Though, with the right lifestyle modifications and certain medications, this condition can be reverted or at least controlled. The reversible particularity of prediabetes has made it a popular target of research, as it may indicate new ways to delay or even avoid the onset of T2DM.

DM chronic hyperglycemia contributes to the onset of many systemic complications such as cardiovascular diseases and hypertension. It can also lead to long-term damage or dysfunction of diverse organs, including eyes, kidneys, nerves, heart and blood vessels, as well as sexual dysfunction [3]. The urgent need to prevent those comorbidities makes DM a popular field of research all over the world.

Impact of diabetes *mellitus* on male fertility

Since the 11th century, researchers have been trying to clarify the bonds between DM and male fertility, describing DM as "a collapse of sexual functions". In the last few years, the deleterious effects of DM on male fertility have been widely investigated due to the increasing incidence in younger individuals [22, 23]. Thus, the notion that DM is usually a disease of elderly population has been disregarded. Nowadays, DM is affecting more and more individuals prior to and during their reproductive years [24, 25], which supports the importance of studying the effects of DM in male reproduction [11].

In the last decade, there was accumulating evidence for the decline in male fertility potential. In fact, the number of couples seeking for medical assistance to have children has increased over the years. Clinically, infertility is defined as the inability to conceive after 1 year of unprotected intercourse [26] and it affects about 13–18% of the couples worldwide [27]. Interestingly, the male factor is the exclusive origin in about one third of all infertility cases [28]. However, there is still a large number of infertility cases (10-20%) with no attributable cause (idiopathic) [29-31], as well as many undiagnosed cases. So, the real numbers of male infertility can be even more disturbing. On the other hand, subfertility describes any form of reduced fertility in couples unsuccessfully trying to conceive [32]. It has been reported that about 50% of male diabetic patients are subfertile [33]. In T1DM patients, it was suggested that spermatogenesis disruption and germ cells apoptosis is related to local autoimmune damage [34]. Besides, approximately 35% of T2DM patients are infertile [34]. Men with T2DM usually present impaired sperm parameters and decreased testosterone serum levels [34]. In fact, the hormonal and metabolic changes induced by DM [35, 36] were proposed as key contributors for the development of male infertility.

Hormonal fluctuations caused by DM affect the glucose sensing mechanisms of testes and spermatozoa, namely the decreased plasma levels of LH and FSH observed in diabetic patients [37-40]. Patients with DM also present a dysregulation of the hypothalamic-pituitary gonadal axis, either due to reduced pituitary sensitivity or an inefficient steroid transport into effector cells [41, 42]. These alterations have a direct effect on the overall testicular functioning, particularly on spermatogenesis, spermiogenesis and spermatozoa metabolism [43, 44]. Besides, the effects induced by DM on testicular functioning are also associated with the lack of insulin [43]. Diabetic individuals commonly use insulin and insulin/analogues for glucose management, however, it is not clear if they are totally effective and safe for the individuals' reproductive health [45-47]. It has been described that insulin stimulates several SCs functions, such as the uptake of free nucleosides, transferrin secretion, DNA and protein synthesis, glycine metabolism and lactate production [48-53], as well as the stimulation of several Leydig cells functions [54], which may affect the outcome of spermatogenesis. Insulin also contributes to the differentiation of spermatogonia into primary spermatocytes, via IGF-I receptor [55], evidencing the need for a tight control of this hormone throughout spermatogenesis. Furthermore, spermatozoa plasma membrane and acrosome are also under the hormonal control of insulin [56]. An *ex vivo* study conducted with human spermatozoa isolated from normozoospermic donors demonstrated that spermatozoa treated with insulin increased their total and progressive motility, acrosome reaction and also nitric oxide production, thus, enhancing spermatozoa fertilization capacity [57]. Moreover, insulin administration in streptozotocin (STZ)-treated diabetic rats completely restored sperm counts and motility [58] and reverted some deleterious effects on epididymis [59]. Ultrastructural alterations in the testis [60], sexual disorders such as erectile dysfunction [61], retrograde ejaculation, impotence or decreased libido [62] are problems commonly observed in diabetic men [63-65]. Recently, it has been proposed that erectile dysfunction is related to IR [66] and that besides human ejaculated spermatozoa secrete insulin, this hormone has a crucial role in the autocrine glucose metabolism regulation [67]. Insulin fluctuations may influence the male reproductive health [67]. It is known that a few hours with insulin deprivation can alter the nutritional support of spermatogenesis [68], including suppressing acetate production [69].

Semen from diabetic individuals presents abnormally high levels of glucose and fructose, and several reports demonstrated ineffective metabolic control in those patients [64]. Moreover, individuals with DM have a defective glucose transport due to a depletion of GLUTs [70]. In fact, sperm glucose uptake and metabolism are crucial endpoints for male fertility potential. Glucose metabolism is a key feature to maintain ATP and adenosine diphosphate (ADP) supplies, essential for spermatozoa function [71, 72]. In the absence of glucose, spermatozoa progressively lose their motility, which is rapidly restored with glucose addition [73], evidencing that glucose concentration is essential to maintain sperm quality. Moreover, sperm hyperactivation and/or capacitation are also highly dependent on glucose [74]. Spermatozoa fertilization potential requires some important alterations such as tyrosine phosphorylation,

hyperactivated motility, calcium movements and acrosome reaction [75, 76]. It was reported that in a medium without glucose there is also an inhibition of spontaneous acrosome reaction, being quickly restored after glucose addition. Thus, glucose homeostasis is very important for the formation of fully competent spermatozoa [77].

Sperm metabolism and DM are intimately related to OS. Diabetic patients usually present ROS overproduction and decreased levels of antioxidant defenses [78], which have an impact in sperm quality: i) decreased sperm motility and viability [79]; ii) increased number of spermatozoa with abnormal morphology [79, 80], iii) reduction in fecundity capacity [81], and iv) irreparable damages in sperm nuclear and mitochondrial DNA [79, 82]. Studies with diabetic male rats also demonstrated a diminished body and reproductive organ weight, lower testicular and epididymal sperm content [59, 83, 84]. Additionally, histological studies revealed a considerable reduction of the seminiferous tubules and epididymal lumen in STZ-treated rats [59]. Apoptosis is another problem resulting from OS [85, 86] that is associated to DM. Actually, it is proposed as one of the main mechanisms to explain the subfertility/infertility detected in diabetic individuals [87]. Still, further studies are needed to understand the impact of DM on male fertility.

References

1. World Health Organization (2018) Diabetes. Available from: https://www.who.int/news-room/fact-sheets/detail/diabetes.
2. Ugochukwu N, et al. (2003) The effect of Gongronema latifolium extracts on serum lipid profile and oxidative stress in hepatocytes of diabetic rats. Journal of biosciences 28(1):1-5.
3. American Diabetes Association (2013) Diagnosis and Classification of Diabetes Mellitus. Diabetes Care, American Diabetes Association, pp. S67
4. Kesavulu M, et al. (2000) Lipid peroxidation and antioxidant enzyme levels in type 2 diabetics with microvascular complications. Diabetes & Metabolism 26(5):387-92.
5. Baliga V and Sapsford R (2009) Diabetes mellitus and heart failure—an overview of epidemiology and management. Diabetes & Vascular Disease Research 6(3):164-71.
6. Hamden K, et al. (2011) Therapeutic effects of soy isoflavones on α-amylase activity, insulin deficiency, liver–kidney function and metabolic disorders in diabetic rats. Natural Product Research 25(3):244-55.
7. Forouhi NG and Wareham NJ (2014) Epidemiology of diabetes. Medicine (Abingdon) 42(12):698-702.
8. Wild S, et al. (2004) Global prevalence of diabetes estimates for the year 2000 and projections for 2030. Diabetes Care 27(5):1047-53.
9. Burul-Bozkurt N, et al. (2010) Diabetes alters aromatase enzyme levels in gonadal tissues of rats. Naunyn-Schmiedebergs' Archives of Pharmacology 382(1):33-41.
10. Grieco FA, et al. (2012) Immunology in the clinic review series; focus on type 1 diabetes and viruses: how viral infections modulate beta cell function. Clinical & Experimental Immunology 168(1):24-9.
11. Agbaje IM, et al. (2007) Insulin dependant diabetes mellitus: implications for male reproductive function. Human Reproduction 22(7):1871-77.
12. Heltianu C, et al. (2011) Genetic Determinants of Microvascular Complications in Type 1 Diabetes. In: D Wagner (ed) Type 1 Diabetes Complications, Tech Open Access Publisher,
13. Berg JM, et al. (2002) Biochemistry. W H Freeman, New York.
14. Carneiro FS, et al. (2010) Erectile dysfunction in young non-obese type II diabetic Goto-Kakizaki rats is associated with decreased eNOS phosphorylation at Ser1177. The Journal of Sexual Medicine 7(11):3620-34.
15. Golay A and Ybarra J (2005) Link between obesity and type 2 diabetes. Best Practice & Research. Clinical Endocrinology & Metabolism 19(4):649-63.

16. DeFronzo RA, et al. (1992) Pathogenesis of NIDDM: a balanced overview. Diabetes Care 15(3):318-68.
17. Edelstein SL, et al. (1997) Predictors of progression from impaired glucose tolerance to NIDDM: an analysis of six prospective studies. Diabetes 46(4):701-10.
18. Centers for Disease Control Prevention (2017) National diabetes statistics report. Atlanta, GA: Centers for Disease Control and Prevention. US Department of Health and Human Services,
19. Rowley WR, et al. (2017) Diabetes 2030: Insights from Yesterday, Today, and Future Trends. Population Health Management 20(1):6-12.
20. Aroda VR and Ratner R (2008) Approach to the patient with prediabetes. Journal of Clinical Endocrinology and Metabolism 93(9):3259-65.
21. Hamman RF (2009) Genetic and environmental determinants of non-insulin-dependent diabetes mellitus (NIDDM). Diabetes/metabolism reviews 8(4):287-338.
22. Lavizzo-Mourey R (2007) Childhood obesity: what it means for physicians. JAMA 298(8):920-2.
23. Harjutsalo V, et al. (2008) Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study. Lancet 371(9626):1777-82.
24. Nguyen RH, et al. (2007) Men's body mass index and infertility. Human Reproduction 22(9):2488-93.
25. Delfino M, et al. (2007) Prevalence of diabetes mellitus in male partners of infertile couples. Minerva Urologica e Nefrologica 59(2):131-5.
26. Zegers-Hochschild F, et al. (2009) International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology, 2009. Fertility and Sterility 92(5):1520-4.
27. Dube E, et al. (2008) Alterations in gene expression in the caput epididymides of nonobstructive azoospermic men. Biology of Reproduction 78(2):342-51.
28. Shukla KK, et al. (2012) Apoptosis, spermatogenesis and male infertility. Frontiers in Bioscience 4:746-54.
29. Seshagiri PB (2001) Molecular insights into the causes of male infertility. Journal of biosciences 26(4 Suppl):429-35.
30. Dohle GR, et al. (2005) EAU guidelines on male infertility. European Urology 48(5):703-11.
31. de Kretser DM (1997) Male infertility. Lancet 349(9054):787-90.
32. Jenkins J, et al. (2004) European Classification of Infertility Taskforce (ECIT) response to Habbema et al.,'Towards less confusing terminology in reproductive medicine: a proposal'. Human Reproduction 19(12):2687-88.
33. La Vignera S, et al. (2009) Andrological characterization of the patient with diabetes mellitus. Minerva endocrinologica 34(1):1.
34. La Vignera S, et al. (2012) Diabetes Mellitus and Sperm Parameters: A Brief Review. Journal of andrology 33(2):145-53.
35. Mallidis C, et al. (2009) The influence of type 1 diabetes mellitus on spermatogenic gene expression. Fertility and Sterility 92(6):2085-7.
36. Bener A, et al. (2009) Is male fertility associated with type 2 diabetes mellitus? International Urology and Nephrology 41(4):777-84.
37. Alves MG, et al. (2013) Diabetes, insulin-mediated glucose metabolism and Sertoli/blood-testis barrier function. Tissue Barriers 1(2):1-10.
38. Distiller LA, et al. (1975) Pituitary responsiveness to luteinizing hormone-releasing hormone in insulin-dependent diabetes mellitus. Diabetes 24(4):378-80.
39. Wright AD, et al. (1976) Luteinizing release hormone tests in impotent diabetic males. Diabetes 25(10):975-7.
40. Bestetti G, et al. (1985) One month of streptozotocin-diabetes induces different neuroendocrine and morphological alterations in the hypothalamo-pituitary axis of male and female rats. Endocrinology 117(1):208-16.
41. Baccetti B, et al. (2002) Insulin-dependent diabetes in men is associated with hypothalamo-pituitary derangement and with impairment in semen quality. Human Reproduction 17(10):2673-7.
42. Dong Q, et al. (1991) Pulsatile LH secretion in streptozotocin-induced diabetes in the rat. Journal of Endocrinology 131(1):49-55.
43. Ballester J, et al. (2004) Insulin-dependent diabetes affects testicular function by FSH-and LH-linked mechanisms. Journal of andrology 25(5):706.
44. Chiodini I, et al. (2006) Hypothalamic-pituitary-adrenal activity in type 2 diabetes mellitus: role of autonomic imbalance. Metabolism: Clinical and Experimental 55(8):1135-40.
45. Cryer PE (2008) The barrier of hypoglycemia in diabetes. Diabetes 57(12):3169-76.
46. Cardoso S, et al. (2011) Impact of STZ-induced hyperglycemia and insulin-induced hypoglycemia in plasma amino acids and cortical synaptosomal neurotransmitters. Synapse 65(6):457-66.
47. Cardoso S, et al. (2010) Cortical and hippocampal mitochondria bioenergetics and oxidative status during hyperglycemia and/or insulin-induced hypoglycemia. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 1802(11):942-51.
48. Griswold MD and Merryweather J (1982) Insulin stimulates the incorporation of 32Pi into ribonucleic acid in cultured sertoli cells. Endocrinology 111(2):661-7.

49. Skinner MK and Griswold MD (1982) Secretion of testicular transferrin by cultured Sertoli cells is regulated by hormones and retinoids. Biology of reproduction 27(1):211-21.
50. Borland K, et al. (1984) The actions of insulin-like growth factors I and II on cultured Sertoli cells. Endocrinology 114(1):240-46.
51. Oonk RB, et al. (1985) Comparison of the effects of insulin and follitropin on glucose metabolism by Sertoli cells from immature rats. Molecular and Cellular Endocrinology 42(1):39-48.
52. Guma FC, et al. (1997) Effect of FSH and insulin on lipogenesis in cultures of Sertoli cells from immature rats. Brazilian Journal of Medical and Biological Research 30(5):591-7.
53. Oliveira PF, et al. (2012) Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells. Biochimica et Biophysica Acta 1820(2):84-89.
54. Perrard-Sapori MH, et al. (1987) Modulation of Leydig cell functions by culture with Sertoli cells or with Sertoli cell-conditioned medium: effect of insulin, somatomedin-C and FSH. Molecular and Cellular Endocrinology 50(3):193-201.
55. Nakayama Y, et al. (1999) IGF-I, IGF-II and insulin promote differentiation of spermatogonia to primary spermatocytes in organ culture of newt testes. International Journal of Developmental Biology 43(4):343-7.
56. Silvestroni L, et al. (1992) Insulin-sperm interaction: effects on plasma membrane and binding to acrosome. Archives of Andrology 28(3):201-11.
57. Lampiao F and du Plessis SS (2008) Insulin and leptin enhance human sperm motility, acrosome reaction and nitric oxide production. Asian journal of andrology 10(5):799-807.
58. Seethalakshmi L, et al. (1987) The effect of streptozotocin-induced diabetes on the neuroendocrine-male reproductive tract axis of the adult rat. Journal of Urology 138(1):190-4.
59. Soudamani S, et al. (2005) Effects of streptozotocin-diabetes and insulin replacement on the epididymis of prepubertal rats: histological and histomorphometric studies. Endocrine Research 31(2):81-98.
60. Cameron DF, et al. (1985) Interstitial compartment pathology and spermatogenic disruption in testes from impotent diabetic men. Anatomical Record 213(1):53-62.
61. De Young L, et al. (2004) Oxidative stress and antioxidant therapy: their impact in diabetes-associated erectile dysfunction. Journal of andrology 25(5):830-6.
62. Schoeffling K, et al. (1963) Disorders of Sexual Function in Male Diabetics. Diabetes 12:519-27.
63. Bartak V (1979) Sperm quality in adult diabetic men. International Journal of Fertility 24(4):226-32.
64. Padron RS, et al. (1984) Semen analyses in adolescent diabetic patients. Acta Diabetologica Latina 21(2):115-21.
65. Ranganathan P, et al. (2002) Sperm cryopreservation for men with nonmalignant, systemic diseases: a descriptive study. Journal of andrology 23(1):71-5.
66. Bansal TC, et al. (2005) Incidence of metabolic syndrome and insulin resistance in a population with organic erectile dysfunction. The Journal of Sexual Medicine 2(1):96-103.
67. Aquila S, et al. (2005) Autocrine regulation of insulin secretion in human ejaculated spermatozoa. Endocrinology 146(2):552-7.
68. Oliveira P, et al. (2012) Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells. Biochimica et Biophysica Acta (BBA)-General Subjects 1820(2):84-89.
69. Alves MG, et al. (2012) In vitro cultured human Sertoli cells secrete high amounts of acetate that is stimulated by 17β-estradiol and suppressed by insulin deprivation. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1823(8):1389-94.
70. Handberg A, et al. (1990) Expression of insulin regulatable glucose transporters in skeletal muscle from type 2 (non-insulin-dependent) diabetic patients. Diabetologia 33(10):625-27.
71. Perchec G, et al. (1995) Relationship between sperm ATP content and motility of carp spermatozoa. Journal of cell science 108(2):747-53.
72. Williams AC and Ford W (2001) The role of glucose in supporting motility and capacitation in human spermatozoa. Journal of andrology 22(4):680.
73. Gorus FK and Pipeleers DG (1986) Glucose metabolism in human spermatozoa: lack of insulin effects and dissociation from alloxan handling. Journal of Cellular Physiology 127(2):261-6.
74. Piomboni P, et al. (2012) The role of mitochondria in energy production for human sperm motility. International journal of andrology 35(2):109-24.
75. Flesch FM and Gadella BM (2000) Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochimica et Biophysica Acta 1469(3):197-235.
76. Harrison RA and Gadella BM (2005) Bicarbonate-induced membrane processing in sperm capacitation. Theriogenology 63(2):342-51.
77. Urner F and Sakkas D (1996) Glucose participates in sperm-oocyte fusion in the mouse. Biology of reproduction 55(4):917-22.
78. Tabak O, et al. (2011) Oxidative lipid, protein, and DNA damage as oxidative stress markers in vascular complications of diabetes mellitus. Clinical and Investigative Medicine. Medecine Clinique et Experimentale 34(3):E163-71.

79. Oliveira PF, et al. (2015) White tea consumption restores sperm quality in prediabetic rats preventing testicular oxidative damage. Reproductive BioMedicine Online 31(4):544-56.
80. Rato L, et al. (2013) High-energy diets may induce a pre-diabetic state altering testicular glycolytic metabolic profile and male reproductive parameters. Andrology 1(3):495-504.
81. Scarano W, et al. (2006) Sexual behaviour, sperm quantity and quality after short-term streptozotocin-induced hyperglycaemia in rats. International journal of andrology 29(4):482-88.
82. Rato L, et al. (2014) Pre-diabetes alters testicular PGC1-α/SIRT3 axis modulating mitochondrial bioenergetics and oxidative stress. Biochimica et Biophysica Acta (BBA)-Bioenergetics 1837(3):335-44.
83. Amaral S, et al. (2006) Effects of hyperglycemia on sperm and testicular cells of Goto-Kakizaki and streptozotocin-treated rat models for diabetes. Theriogenology 66(9):2056-67.
84. Hassan AA, et al. (1993) The effect of diabetes on sexual behavior and reproductive tract function in male rats. Journal of Urology 149(1):148-54.
85. Grunewald S, et al. (2008) Relationship between sperm apoptosis signalling and oocyte penetration capacity. International journal of andrology 31(3):325-30.
86. Aitken RJ and Koppers AJ (2011) Apoptosis and DNA damage in human spermatozoa. Asian journal of andrology 13(1):36-42.
87. Roessner C, et al. (2012) Sperm apoptosis signalling in diabetic men. Reproductive Biomedicine Online 25(3):292-9.

Nutrition, antioxidants and male reproduction

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- **Dias TR**, Bernardino RL, Meneses MJ, Sousa M, Sá R, Alves MG, Silva BM, Oliveira PF. (2016) "Emerging potential of natural products as an alternative strategy to pharmacological agents used against metabolic disorders". Current Drug Metabolism 17(6):582-597 (DOI:10.2174/1389200217666160229113629).

- **Dias TR**, Alves MG, Casal S, Oliveira PF, Silva BM. (2017) "Promising potential of dietary (poly)phenolic compounds in the prevention and treatment of diabetes *mellitus*" Current Medicinal Chemistry 24(4):334-354 (DOI:10.2174/0929867323666160905150419).

- **Dias TR**, Carrageta DF, Alves MG, Oliveira PF, Silva BM. (2018) "White tea" In: Seyed Nabavi and Ana Silva (eds), Nonvitamin and Nonmineral Nutritional Supplements, 1st edition, Academic Press, Elsevier, pp. 437-445 (DOI: 10.1016/B978-0-12-812491-8.00058-8), (ISBN:9780128124918).

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Nutrition and male reproductive potential

Nutrition is a primitive need essential for human survival and reproduction. The dietary patterns have changed along the years, especially with industrialization and technology that directly or indirectly controlled the food market and promoted a sedentary lifestyle. Over-processed products containing artificial sweeteners, preservatives and refined sugars gained ground over the organic ones. The over-processed foods are usually produced in a larger scale, making them cheaper and more accessible to everyone. Additionally, multi-million-dollar marketing strategies make those products very attractive, particularly to young people. But not all the alterations of modernization were unfavorable. It also brought increased knowledge and education about nutrition, a higher support by health care systems and the promotion of a healthy lifestyle. However, in general, people do not just eat what the body requires to function, they consume bigger portions than they should because eating also gives a sense of relaxation, comfort and pleasure. So, individuals are consuming more and wasting fewer calories. This overeating scenario, together with the lack of physical activity, contributed to the alarming proportions of several diseases worldwide, including DM and obesity. On the other hand, there are many countries where food is not so abundant, and people are undernourished, which also triggers the development of severe diseases. Even so, according to the WHO, in many countries overnutrition is now killing more people than undernutrition. However, both malnutrition types contribute to the increasing incidence of diet-related diseases year by year.

The impact of diet and other daily life activities in male reproductive potential is currently a matter of debate among researchers. In fact, the nutritional status of an individual is of great importance to maintain the well-functioning of the whole body, which may have an impact in the reproductive function. Many minerals, vitamins and other food components obtained through the diet have demonstrated an essential role in processes involved in spermatozoa production and survival. Particularly, there are many dietary compounds with antioxidant properties that are essential for sperm protection from oxidative damages. Modern societies are currently aware of the benefits of having an adequate nutrition and practicing regular physical activity, and how these factors can influence important aspects of people's life. Although this so called "healthy lifestyle" may vary among cultures, it has been associated with the prevention of many diseases, which led many people to change their daily habits. However, great part of the population still devalues the importance of having a well-balanced diet. On the other hand, it is difficult to promote a healthier lifestyle when there are so many attractive fast food restaurants to try out. For instance, in the USA there are over 200 000 fast food restaurants with a huge diversity of concepts and meals, leading countless Americans to eat fast food daily. Moreover, millions of vending machines containing soft drinks, sweets and refined foods are available pretty much everywhere, thus reflecting the actual tastes and choices of consumers. The scenario is even most worrying because children and adolescents are the most attracted ones. Generally, people are on a rush, thus making bad food choices, eating

too fast, alone and less frequently or even resort to food supplements to save time. This situation usually leads to nutritional deficiencies, whether of one or more nutrients, having negative outcomes for human health. Additionally, there are many jobs with a sedentary nature or that require exposure to high temperatures or toxic compounds during long periods. Furthermore, couples are so focused on their professional careers that are postponing the age to have children, and this could be a major obstacle to achieve conception.

The current dietary and lifestyle routines make people more susceptible to develop pathological conditions, including male subfertility/infertility. Among the most prevalent diet-related diseases in the world are DM, obesity, cardiovascular diseases, cancer and infections, and all these conditions have been linked with a decreased male fertility potential [1]. OS has been reported as a major contributing factor for the development of those diseases, but it can also be a result of the unhealthy status created by the disease itself. This kind of stress occurs when the production of free radicals overwhelms the ability of inner antioxidant defenses to control them. OS is usually found in subfertile and infertile men, thus having a key role on the pathophysiology of male subfertility/infertility. In fact, high levels of free radicals were detected in the semen of 25-40% of infertile men, as well as a lower amount of inner antioxidant enzymes than in fertile men [2]. Antioxidant compounds obtained through the diet may be of extreme relevance to restore the reproductive tract antioxidant defense system and avoid fertility dysfunctions induced by OS.

Oxidants, antioxidants and sperm function

A free radical is a chemical compound with one or more unpaired electron(s) in an external orbital, which makes it unstable and highly reactive. The term ROS usually refer to oxygen-containing free radicals, such as superoxide anion ($O_2^{\bullet -}$), hydroxyl ($^{\bullet}OH$) and nitric oxide ($NO^{\bullet}$) radicals, but also to some non-radical oxygen derivatives, including singlet oxygen ($^1O_2$), ozone (O_3), hydrogen peroxide (H_2O_2), peroxynitrite anion ($ONOO^-$) and hydrogen hypochlorite (HClO). These latter do not contain unpaired electrons but are potentially reactive and can be converted to radical ROS. Cellular endogenous sources of ROS, mostly generated by oxidative phosphorylation in mitochondria or by the immune system, have an essential role in normal cellular processes. In physiological conditions, ROS are involved in the regulation of cell signaling pathways, having the ability to modulate the activity of enzymes, mediate inflammation and control pathogens intrusion. For instance, SCs require ROS for normal metabolic processes, namely to obtain energy and produce important substrates for germ cells [3]. Moreover, spermatozoa also need physiological level of ROS for essential fertilizing processes, such as sperm capacitation, hyperactivation, acrosome reaction and sperm-egg fusion. However, when something disrupts the prooxidant-antioxidant homeostasis in those cells, an oxidative environment can be created, impairing spermatogenesis and sperm function. Defective spermatozoa (immature or abnormal), high levels of leukocytes present in the

ejaculate (leukocytospermia), pathological conditions or other exogenous factors (e.g. smoke, radiation or drugs), are major stimuli for ROS overproduction (Figure 1.6). The most common ROS produced in spermatozoa and that can harm their quality are superoxide anion, hydrogen peroxide, nitric oxide and peroxynitrite. Proteins, lipids, and DNA are the core targets of ROS as they can capture electrons from them to seek stability. However, this process triggers a cascade of reactions due to the generation of new unstable species that will subsequently try to capture electrons from a nearby molecule as well. These events lead to protein oxidation, lipid peroxidation and/or oxidation of DNA bases, which culminates in severe cellular damages and even cell death.

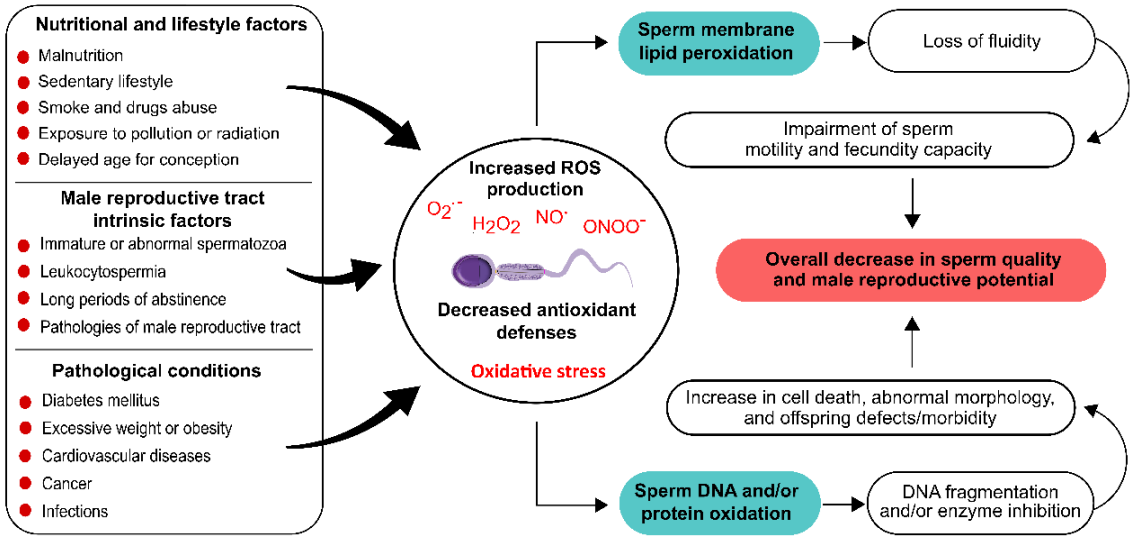


Figure 1.6 Oxidative stress-promoting factors in spermatozoa and the respective outcomes for male reproductive function. Nutrition, lifestyle and pathological conditions are among the extrinsic factors promoting oxidative stress in the male reproductive tract. There are also some intrinsic factors such as immature/abnormal spermatozoa and leukocytospermia that may increase ROS production within the male reproductive tract, including in spermatozoa. Superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), nitric oxide radical ($NO^{\bullet}$) and peroxynitrite anion ($ONOO^-$) are the main types of ROS produced by spermatozoa. ROS overproduction together with the decrease in antioxidant defenses may lead to sperm membrane lipid peroxidation and and/or oxidation of DNA bases. While lipid peroxidation alters the fluidity of spermatozoa membrane, impairing motility and fecundity, DNA oxidation results in DNA fragmentation, thus increasing cell death, the number of abnormal spermatozoa and the occurrence of defects/morbidity in the offspring. This culminates in the overall decrease in sperm quality and impairment of male reproductive potential.

Spermatozoa are quite susceptible to oxidative damages due to their distinctive lipidic membrane and limited intracellular defense system. When ROS attack the PUFAs-rich membrane of spermatozoa, lipid radicals are formed, which in the presence of oxygen generate lipid peroxyl radicals. Those products contribute to the formation of some dangerous adducts, such as the 4-hydroxynonenal (4-HNE) or malondialdehyde (MDA), that stimulate a chain reaction of free radicals' generation usually described as lipid peroxidation. Consequently, spermatozoa membrane loses fluidity, which compromises motility and sperm-egg fusion. ROS can also inactivate key enzymes involved in sperm function by altering their structural

conformations. Moreover, ROS-induced damages to mitochondrial and nuclear sperm DNA may end-up in low pregnancy rates, increased birth defects and high offspring morbidity.

Generally, antioxidants are compounds with the ability to scavenge ROS or act as cofactors for the cellular enzymatic antioxidant defenses, thus preventing oxidative-induced damages. To counteract these severe damages, spermatozoa are endowed by an intrinsic antioxidant system that contains a myriad of enzymatic and non-enzymatic components, including superoxide dismutase, catalase, glutathione peroxidase, glutathione transferases, peroxiredoxins and thioredoxins. Still, the amount of those scavenging compounds in spermatozoa cytoplasm is very low. Chromatin condensation is one of the vital processes occurring during spermatogenesis, in which most of sperm DNA histones are replaced by protamines to obtain a higher compaction and protection. If these events are disrupted and spermatozoa retain more histones than they should, sperm DNA becomes more susceptible to oxidative damages, which is critical because sperm DNA has a low ability for repair. On the other hand, during spermiation, most of sperm cytoplasm and organelles are removed, thus diminishing sperm endogenous defenses. Sometimes, this process is not complete, and some spermatozoa retain a residual cytoplasmic droplet, which contains enzymes that promote ROS production. Nevertheless, epididymal fluid and seminal plasma possesses a powerful antioxidant system that protects spermatozoa from OS. These fluids contain many enzymatic antioxidants, namely superoxide dismutase, catalase and glutathione peroxidase. However, they also contain many non-enzymatic antioxidants that can be obtained through the diet, supporting the key role of nutrition in the control of reproductive fluids composition and antioxidant capacity. Thus, the nutritional state of a man really influences his fertility potential.

Natural products in the treatment of diabetes *mellitus*

The primary strategy to counteract DM includes nutritional changes and physical activity. However, these interventions are usually unsuccessful and need to be complemented with medication. Over the last decades, pharmaceutical industry has developed several drugs that are currently used in the treatment of DM or its associated complications. The global healthcare expenditure in preventing and treating this metabolic disorder is colossal. In 2015, the total annual costs in USA associated with DM diagnosed cases was about 408 billion USD, and it is estimated to increase to 622 USD in 2030 [4]. Conventionally, T1DM is treated with exogenous insulin [5] and T2DM with synthetic oral hypoglycemic agents, such as sulfonylureas and biguanides [1]. Although these drugs are effective in reducing glycemia, many of them fail as a curative agent for diabetic complications and have serious adverse effects such as weight gain, hypoglycemia, edema and gastrointestinal disturbances that can discourage patients' compliance. Besides, they have high development costs. This enforces the need to search for more efficient and innovative therapies and the market for antidiabetic drugs is potentially huge.

For thousands of years, natural products have played an important role in treating and preventing human diseases. Traditional herbal medicines, derived predominantly from plants, have aroused considerable interest in recent years [6], and their potentialities for drug discovery have long been recognized by pharmaceutical industry. The increasing predominance of more advanced drug discovery methods, such as molecular approaches, led to a decline in the study of natural products. However, they are still providing their fair share as new clinical candidates and drugs. For instance, 19 natural-related drugs were approved for marketing worldwide from 2005-2010 [7]. Natural products have demonstrated promising antioxidant, antimitotic, antimicrobial, anti-inflammatory, antiangiogenic, antidiabetic and anticarcinogenic properties, which may be of great importance in the treatment of human diseases. Moreover, there are a multitude of plants with insulin mimetic or insulin secretory activity, hypoglycemic or anti-hyperglycemic potential, capacity to increase glucose utilization or glucose uptake by cells and combat secondary complications [8, 9]. Natural compounds are considered less toxic and relatively cheaper than synthetic ones and large amounts can be potentially consumed in everyday diet [10, 11]. Therefore, the search for more effective and safer hypoglycemic agents has become one important area of investigation [12].

In living organisms, the ROS levels are controlled by inner antioxidant defense system. However, these intrinsic antioxidants have a limited ability to counteract free radicals. In most cases, they can minimize, but not completely prevent, oxidative damage to biomolecules, eventually leading to disease [13]. Therefore, exogenous antioxidants with the ability to scavenge free radicals may be of great value in the prevention of the onset and/or progression of human diseases, including DM [14]. Although plants are nutritional sources of macroconstituents as carbohydrates, lipids, proteins and fibers, they are also a rich source of antioxidants. Human diet is very rich in phenolic compounds, which are the most abundant natural antioxidants [15]. There is compelling evidence reporting the benefits of the long-term consumption of phenolics in the prevention of several OS-induced diseases, such as prediabetes and DM [16, 17]. It has been shown that phenolics can modulate metabolic enzymes, nuclear receptors, gene expression and multiple signaling pathways [18].

Among the several thousands of phenolic compounds that have already been identified in the Plant Kingdom, only a limited number is significantly present in human diet. Besides, phenolic compounds are almost ubiquitous in edible and medicinal plants. Although their diversity makes it difficult to estimate the total content of phenolic compounds in foods, it has been demonstrated that it may be as high as 150 g/kg (in cloves) [19]. Spices, fruits, seeds and vegetables are among the richest dietary sources of phenolic compounds. Additionally, there are some beverages that highly contribute to the daily intake of phenolics: coffee (200-550 mg of phenolics/cup), tea (150-200 mg of phenolics/cup) and wine (200-800 mg of phenolics/glass) [20, 21]. Other phenolic-rich products, include cereals, cocoa products and olive oil [19]. There are so many phenolic-rich foodstuffs that it is difficult to follow a diet totally free of these phytochemicals. Due to the individual food preferences and country-specific dietary patterns,

there is a high variability on the daily content of consumed phenolics. However, it has been reported that people following a diet containing several servings of fruit and vegetables per day, as well as coffee-rich or tea-rich beverages commonly reach a total phenolic intake of 1 g per day [22, 23]. This is a much higher amount in comparison to all the other classes of antioxidants, including vitamin C and E [24].

Phenolic compounds are a complex class of naturally-occurring bioactive molecules that result from plants secondary metabolism, more specifically from shikimate and acetate pathways [25]. These phytochemicals are essential to plant pigmentation, growth and reproduction, but they also act as a defense system against ultraviolet radiation, oxidants, and aggression by pathogens [26]. Generally, phenolic compounds with only one phenolic ring are considered simple phenols, while those with more than one phenolic ring are designated as polyphenols. More specifically, considering the number of phenolic rings that they contain and the structural elements that bind these rings to one another, phenolic compounds can be classified into four main classes: phenolic acids (simple phenols), stilbenes, lignans and flavonoids (polyphenols) (Annex 1 - Supplementary figure 1) [27].

Tea

Tea is one of the most widely consumed beverages in the world, next to water [28, 29] with a per capita consumption of approximately 120 mL/day [30]. This popularity is probably related with its sensorial properties, relatively low retail price, stimulating effects and potential health benefits [31, 32]. Despite tea is largely drunk for pleasure, its medicinal effects have been widely investigated, having a long, rich history with its first references nearly 5,000 years ago [33]. Tea is prepared as an infusion with the leaves of *Camellia sinensis* (L.), a plant cultivated in over 30 countries across the world that belongs to the Theaceae family [34]. There are two main varieties of tea plants: i) *C. sinensis* var. sinensis, a small-leaved, bush like plant originating from China, which grows in several countries of Southeast Asia experiencing a cold climate; and ii) *C. sinensis* var. assamica, a large-leaved tree discovered in the Assam region of India, which grows in several countries with a semitropical climate [35]. Different types of tea can be obtained from *Camellia sinensis*: white, green, oolong and black teas [36]. The distinguishing factor that defines the different types of tea is the "level of fermentation" of the leaves (or buds) during manufacturing [35]. This process is more accurately called "oxidation", as it consists in the degree of enzymatic oxidation that is allowed to occur from the freshly picked leaves until dryness [36]. White tea (WTEA) is considered the less processed tea type, followed by green tea (GTEA). These non-fermented or very-light fermented tea types are very rich in polyphenolic flavonoid-derived compounds known as catechins (flavan-3-ols) [37]. On the other hand, during oolong and black tea processing, the catechins present in tea leaves are oxidized and polymerized, by polyphenol oxidase, forming yellow-orange pigments called theaflavins (dimers) and thearubigins (oligomers) [38, 39]. These compounds contribute

not only for the color, but also for the bitterness and astringency of oolong and black teas [40]. Although theaflavins have demonstrated a similar free radical scavenging activity as catechins in certain biological processes [41], catechins are usually more potent antioxidants than theaflavins and thearubigins [42]. Catechins demonstrated potent antioxidant potential acting as reducing agents, singlet-oxygen quenchers, and metallic-ion chelators [43]. Hereupon, the amount of catechins is positively correlated with the antioxidant potential. Therefore, WTEA has become more attractive in recent years due to its high antioxidant activity. An obvious evidence of this scenario is that many publications from the last two decades, reporting the total amount of tea produced and consumed in the world, did not include WTEA [44, 45]. However, statistical data of tea consumption in 2015 in the USA, indicated a consumption of 85% of black tea, 14% of GTEA and the remaining 1% of oolong and WTEA [46]. Thus, WTEA does not seem very popular among tea consumers but is gaining ground. Unfamiliarity, controversial results, its subtle taste and higher cost may be some of the reasons for this low WTEA consumption. Though, the beneficial health effects of WTEA have received great attention among researchers in recent years.

White tea

In contrast to the other tea types, there is no general accepted definition of WTEA. There is still great controversy concerning the manufacture and its content in caffeine and catechins. WTEA is best known in Asia and less in western communities that prefer black tea. Nevertheless, in Europe, WTEA flavor seems to be more accepted than that of GTEA [47]. In fact, the availability of WTEA in European supermarkets has been increasing, which is certainly the response to a higher demand from consumers. WTEA is characterized by a very pale-yellow color and a mild delicate and sweet taste. Though, there are also some versions with added flavors. Annually, very small quantities of WTEA are produced, as it is prepared from new unopened buds of the tea plant and/or immature leaves covered with silvery-white tiny hairs [48], which is the reason why it is called “white” tea. In addition, the harvesting should be made only once a year in the early spring to attain a higher quality. Traditionally, after the plucking, the buds/leaves are gentle spread out to dry under the sun, in a quite long process, to ensure the maintenance of the leaf structure and avoiding any breaks by curling or twisting [49]. During drying, the tea becomes slightly “oxidized”, as it contains very small amounts of theaflavins and thearubigins [39]. Thus, it is wrongly called a non-fermented tea, and it should be considered a very-light fermented tea instead [50]. Nevertheless, there are some variations among the processing techniques that lead to different WTEA types and should be taken in consideration. One of the most known and expensive WTEA types is the Bai Hao Yin Zhen, also known as Silver Needle. It is produced in the Fujian province in China using only new leaf shoots, preferably harvested by hand [51]. The processing method for the production of Silver Needle includes a one-day drying of the buds under the sun, on sieves or drying mats, followed by

baking over a slow fire until fully remove the moisture content [49]. Bai Mu Dan or White Peony is another type of WTEA from the Chinese Fujian province, which uses one leaf shoot and two young leaves, thus attaining a light golden-brown color when brewed and a more intense taste relative to Silver Needle [51]. The manufacture of White Peony includes a withering process under the sun (during 1-3 days), which is followed by drying in a basket [49].

Generally, the main bioactive constituents of WTEA include free amino acids, methylxanthines, and phenolic compounds. The most prevalent free amino acid in tea is L-theanine (Figure 1.7), representing about 4% of leaves dry weight. L-theanine contributes to the pleasant and relaxing effects of tea [52]. Concerning methylxanthines, the most abundant in tea is caffeine (about 3.5%), but it also contains low amounts of theobromine (0.15–0.2%) and theophylline (0.02–0.04%) [50]. Caffeine (Figure 1.7) popularity is attributed to its potent stimulating properties and is usually associated with coffee or caffeinated energy drinks. However, the amount of caffeine among tea types has also been questioned. There are many reports stating that WTEA has the less content in caffeine among the several tea types [53, 54]. Though, this affirmation cannot be so strictly made due to the above-mentioned variables. Hence, other parameters should be used to distinguish WTEA and GTEA, such as the color, the taste, the plucking and the processing steps.

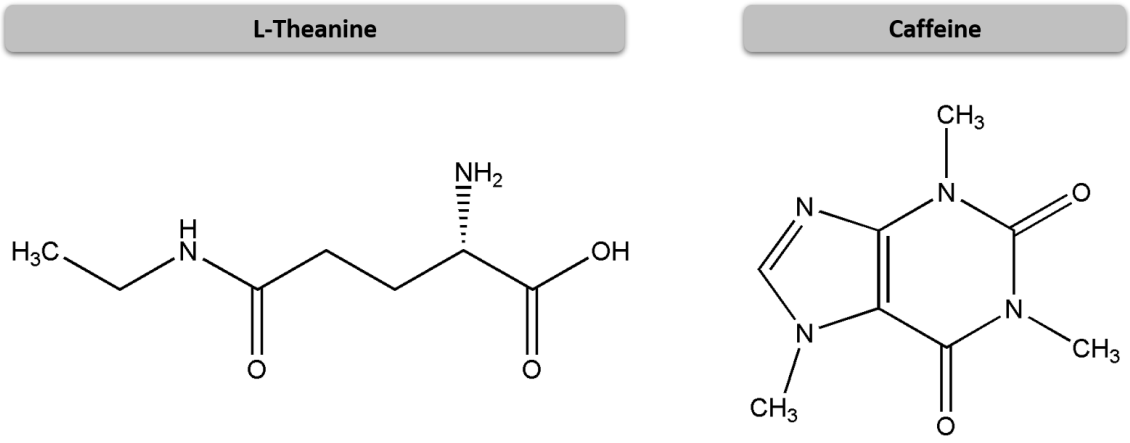


Figure 1.7 Chemical structures of L-theanine and caffeine, which are abundant components in white tea. L-theanine is responsible for the relaxing properties of tea, while caffeine has potent stimulating properties.

Among phenolic compounds, the catechin content really stands out, representing more than 20-30% of the leaves dry weight [39]. These are the compounds to which are attributed most of WTEA antioxidant properties. The main catechin derivatives present in WT are: epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCC) (Figure 1.8). This latter accounts for about 50% of all the catechins and is considered the most bioactive component of tea [39]. The most effective radical scavengers in WTEA are EGCG and EGC due to their characteristic structure (Figure 1.8) [37]. While both EGCG and EGC present hydroxyl (OH) groups at positions 3', 4' and 5' of the B-ring, EGCG has also a gallate moiety

esterified at carbon 3 of the C-ring [37]. The catechin content is often mistakenly used as a differentiation parameter between WTEA and GTEA, but it is not a reasonable approach. There are some WTEA types that contain a higher catechin content than GTEA [39, 55], but the opposite also occurs [55]. This is due to the fact that tea chemical composition is influenced not only by differences in the processing techniques, but also by the geographical origin, climate, soil, botanical variety, harvest time, horticultural practices, and even brewing conditions [35, 49]. Thus, there is a high variance not only in the catechin content, but also in other components, such as methylxanthines. On the other hand, it does seem feasible to use catechin content as a differentiating parameter when comparing either WTEA or GTEA with oolong and black tea [39]. Indeed, most of black tea content are thearubigins (60-70%), remaining only 3-10% of catechins [56].

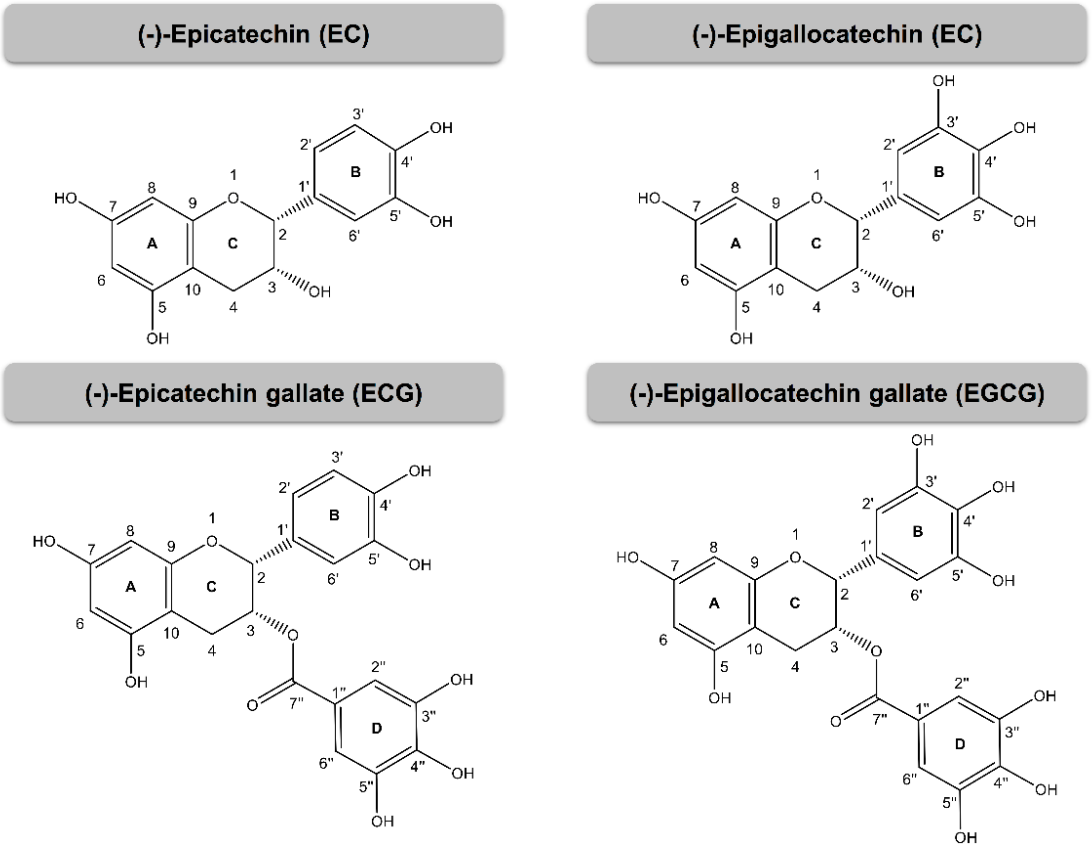


Figure 1.8 Chemical structures of the main catechins present in white tea: (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG). The strong antioxidant potential of catechins is related to the higher number of phenolic rings and hydroxyl groups (OH). The presence of the catechol group (3',4'-ortho-dihydroxylation) in the B-ring, 3-hydroxyl group in the C-ring; and the 5,7-meta-dihydroxylation in the A-ring are key structural features that improve catechins' antioxidant activity.

Health benefits of white tea

Most people drink tea not only for its sensorial characteristics, but also for the variety of health benefits associated to it. Most of WTEA health-promoting effects are attributed to its high

content in phenolic compounds and respective antioxidant activity [38, 39]. As WTEA is rich in catechins, it could be a cost-effective agent to maintain the redox balance in certain human health conditions. In fact, studies with animal models subjected to the ingestion of a WTEA demonstrated an increase in the antioxidant potential of different organs, such as the heart [57], lungs [58], and brain [59]. Moreover, data from clinical trials showed that a single dose of tea (2 g tea solids in 300 ml water) improves plasma antioxidant capacity of healthy adults within 30 to 60 minutes after ingestion [60]. Interestingly, although the benefits of tea are mainly attributed to catechins, there are some studies defending that the combined effect of all the tea components is more therapeutically effective [61]. For instance, there are reports on the compensatory relaxing effect of L-theanine against the stimulatory effect of caffeine [62].

The anti-diabetic potential of tea components and specifically of WTEA has been reported. *In vitro* studies showed that WTEA extracts (0.5 g of dry leaves in 20 mL of hot water) have a stronger hypoglycemic and hypolipidemic potential relative to GTEA and black tea extracts [63]. Moreover, STZ-induced diabetic rats fed with a WTEA aqueous extract (0.5% (w/v)) during 4 consecutive weeks, demonstrated a decrease in blood glucose concentration, improvement in glucose tolerance, and decrease in total serum cholesterol [64]. Furthermore, WTEA may be a good complementary or alternative treatment against obesity and its associated complications. In fact, tea catechins (100 mg in 3 mL fat emulsion), especially ECG and EGCG, have demonstrated an effective role in reducing cholesterol absorption from intestine, thus lowering its solubility and enhancing its excretion [65]. They also reduced total serum cholesterol [64], body weight gain, visceral and liver fat accumulation, and the development of hyperinsulinemia and hyperleptinemia [66]. In addition to catechins, caffeine and L-theanine demonstrated a suppressive effect on body weight increase and fat accumulation [67]. The anti-obesity potential of tea components seems to be largely due to a synergistic effect between catechins and caffeine [67]. Moreover, an *in vitro* study using human pre-adipocytes indicated that WTEA has strong lipolytic and anti-adipogenic activities [68]. Thus, WTEA consumption may reduce adipose tissue size and stimulate weight loss.

Both DM and obesity constitute a risk factor for the development of hypertension and cardiovascular diseases [69]. So, it is of extreme relevance to find new therapeutic approaches to prevent the progression of human disorders to severe complications and WTEA looks quite promising. Tea polyphenols showed an anti-thrombogenic and anti-inflammatory action [70]. Polyphenols may also have a vasculoprotective action and inhibit lipid oxidation [71]. As the oxidation of low and very low-density lipoproteins (LDL and VLDL) can cause the progressive obstruction of arteries or atherosclerosis, leading to coronary heart disease infarction [72], WTEA can be an attenuating factor for the progress of such diseases. Additionally, the most prevalent amino acid present in WTEA, L-theanine, has been described as a blood pressure reducing agent [73]. The replacement of water intake by *ad libitum* WTEA during two months

by a prediabetic animal model demonstrated cardioprotective effects through the improvement of cardiac tissue metabolism [57].

In the central nervous system, OS is one of the main factors contributing to aging processes and neurodegenerative diseases, such as Alzheimer's, Parkinson's, or Huntington's diseases [74, 75]. Polyphenols have demonstrated a neuroprotective role due to its potent antioxidant activity [76]. In addition, L-theanine also induces a relaxation feeling by lowering cortisol levels and reducing psychological and physiological stress [77]. Prediabetic rats drinking WTEA in *ad libitum* conditions during two months (in replacement to water intake), showed an improvement in the metabolic and oxidative profile of cerebral cortex [59], which could be of great value to prevent the development of neurodegenerative disorders.

OS also plays a preponderant role in the development of cancer. It may lead to DNA damage, which culminate in an uncontrolled cell division. DNA mutations are a critical step in carcinogenesis and elevated levels of oxidative-induced DNA lesions have been described in many tumors [78]. WTEA revealed important anticarcinogenic [79] and antimutagenic [80] activities. In fact, it has been reported that WTEA has an antiproliferative effect against cancer cells, while protecting normal cells against DNA damage due to its strong antioxidant activity [79]. Additionally, non–small cell lung cancer cells apoptosis was induced after exposure to a WTEA extract through the modulation of peroxisome proliferator-activated receptor-γ and the 15-lipoxygenase signaling pathways [81]. Thus, WTEA consumption may have antineoplastic and chemopreventive effects that could be vital in the prevention of cancer.

WTEA extracts also exhibited strong antimicrobial and antifungal activities, which were attributed to the polyphenolic content [39, 50]. For instance, catechins were reported to reduce *Escherichia coli* growth in about 50% [82]. EGCG seems to be the stronger antimicrobial catechin. However, it has been reported that the bactericidal effect of EGCG is less effective for gram-negative bacteria than for gram-positive bacteria, as this latter can absorb higher amounts of EGCG [83]. Tea catechins also demonstrated the ability to inhibit human immunodeficiency virus (HIV) propagation by inhibiting the enzyme reverse transcriptase [84]. Altogether, WTEA comprises antimicrobial, antifungal and antiviral activity, which highlights its potential against infections. Besides, the synergistic effects between tea polyphenols and antibiotics has been explored [39]. Interestingly, the antibacterial effect ampicillin has been maximized when in combination with WTEA extracts, possibly because they directly or indirectly attack the same binding site on the bacterial surface [39].

Recently, the beneficial effects of WTEA to prevent skin aging have been investigated [85]. WTEA extract exhibited a protective role on human dermal fibroblast cells, due to its antioxidant and anti-inflammatory activity [86]. WTEA extract (2% in propylene glycol : ethanol : water (5 : 3 : 2)) was described as an effective anti-wrinkle agent, as it was able to decrease epidermal thickness and increase collagen and elastic fiber content in mice suffering from

ultraviolet-induced photoaging [87]. Furthermore, clinical trials also demonstrated that the topical application of WTEA extract may provide protection from ultraviolet radiation-induced Langerhans cell and DNA damage, which could lead to suppression of the immune system and development of skin cancer [88]. Thus, WTEA oral or topical administration may have an anti-aging effect and improve the health of skin [89].

Overall, WTEA and its interesting components may be of great value in the prevention and treatment of several human health conditions. Many studies demonstrate the higher therapeutic potential of WTEA when compared to the other tea types. Though, after tea ingestion, low plasma concentrations of phenolic compounds are attained [90]. Thus, the development of tea-based food supplements can be an alternative. In modern societies, the use of supplements is becoming a trend. In 2015, the global annual sales of dietary supplements exceeded 120 billion USD and are expected to increase to 278 billion USD by 2024 [91]. There is a vast range of over-the-counter supplements in the market, including vitamins, minerals, and botanicals [92]. GTEA preparations are among the top selling botanical dietary supplements, especially for anti-carcinogenic [93], weight loss [94] and skin anti-aging purposes [95]. Moreover, as no undesired effects have been found with tea ingestion, people are eager to try tea-based products to potentiate its health-promoting effects. The development of WTEA-based food supplements could be a promising approach to enhance the beneficial effects of WTEA and improve human health.

References

1. Dias TR, et al. (2016) Emerging potential of natural products as an alternative strategy to pharmacological agents used against metabolic disorders. Current drug metabolism 17(6):582-97.
2. Agarwal A, et al. (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. Fertility and sterility 79(4):829-43.
3. Dias TR, et al. (2013) Glucose Transport and Metabolism in Sertoli Cell: Relevance for Male Fertility. Current Chemical Biology 7(3):282-93.
4. Rowley WR, et al. (2017) Diabetes 2030: Insights from Yesterday, Today, and Future Trends. Population Health Management 20(1):6-12.
5. Association Diabetes Association (2013) Diagnosis and classification of diabetes mellitus. Diabetes care 36(Supplement 1):S67-S74.
6. Ayyanar M, et al. (2008) Traditional herbal medicines used for the treatment of diabetes among two major tribal groups in south Tamil Nadu, India. Ethnobotanical leaflets (1):32.
7. Mishra BB and Tiwari VK (2011) Natural products: an evolving role in future drug discovery. European journal of medicinal chemistry 46(10):4769-807.
8. Bhushan MS, et al. (2010) An analytical review of plants for anti diabetic activity with their phytoconstituent & mechanism of action. International Journal of Pharmaceutical Sciences and Research 1(1):29-46.
9. Patel D, et al. (2012) An overview on antidiabetic medicinal plants having insulin mimetic property. Asian Pacific journal of tropical biomedicine 2(4):320-30.
10. Saxena A and Vikram NK (2004) Role of selected Indian plants in management of type 2 diabetes: a review. The Journal of Alternative & Complementary Medicine 10(2):369-78.
11. Coman C, et al. (2012) Plants and natural compounds with antidiabetic action. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 40(1):314-25.
12. Malviya N, et al. (2010) Antidiabetic potential of medicinal plants. Acta Poloniae Pharmaceutica 67(2):113-18.

13. Ceriello A and Motz E (2004) Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. Arteriosclerosis, thrombosis, and vascular biology 24(5):816-23.
14. Evans JL, et al. (2002) Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. Endocrine reviews 23(5):599-622.
15. Del Rio D, et al. (2013) Dietary (poly) phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxidants & redox signaling 18(14):1818-92.
16. Pandey KB and Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. Oxidative medicine and cellular longevity 2(5):270-78.
17. Arts IC and Hollman PC (2005) Polyphenols and disease risk in epidemiologic studies. The American journal of clinical nutrition 81(1):317S-25S.
18. Dembinska-Kiec A, et al. (2008) Antioxidant phytochemicals against type 2 diabetes. British Journal of Nutrition 99(E-S1):ES109-ES17.
19. Pérez-Jiménez J, et al. (2010) Identification of the 100 richest dietary sources of polyphenols: an application of the Phenol-Explorer database. European Journal of Clinical Nutrition 64(Suppl 3):S112-S20.
20. Bravo L (1998) Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutrition reviews 56(11):317-33.
21. Lakenbrink C, et al. (2000) Flavonoids and other polyphenols in consumer brews of tea and other caffeinated beverages. Journal of agricultural and food chemistry 48(7):2848-52.
22. Manach C, et al. (2004) Polyphenols: food sources and bioavailability. The American journal of clinical nutrition 79(5):727-47.
23. Wang PY, et al. (2016) Higher intake of fruits, vegetables or their fiber reduces the risk of type 2 diabetes: A meta-analysis. Journal of Diabetes Investigation 7(1):56-69.
24. Scalbert A, et al. (2005) Polyphenols: antioxidants and beyond. The American journal of clinical nutrition 81(1):215S-17S.
25. Herrmann KM (1995) The shikimate pathway as an entry to aromatic secondary metabolism. Plant Physiology 107(1):7.
26. Beckman CH (2000) Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? Physiological and Molecular Plant Pathology 57(3):101-10.
27. Almeida S, et al. (2016) Are Polyphenols Strong Dietary Agents Against Neurotoxicity and Neurodegeneration? Neurotoxicity research:1-22.
28. Cheng TO (2004) Will green tea be even better than black tea to increase coronary flow velocity reserve? American Journal of Cardiology 94(9):1223-23.
29. Vinson JA (2000) Black and green tea and heart disease: a review. Biofactors 13(1-4):127-32.
30. McKay DL and Blumberg JB (2002) The role of tea in human health: an update. Journal of the American College of Nutrition 21(1):1-13.
31. Baptista JAB, et al. (1998) Comparison of catechins and aromas among different green teas using HPLC/SPME-GC. Food Research International 31(10):729-36.
32. Baptista JAB, et al. (1999) Comparative study and partial characterization of Azorean green tea polyphenols. Journal of Food Composition and Analysis 12(4):273-87.
33. Wheeler DS and Wheeler WJ (2004) The medicinal chemistry of tea. Drug development research 61(2):45-65.
34. López V and Calvo MI (2011) White tea (Camellia sinensis Kuntze) exerts neuroprotection against hydrogen peroxide-induced toxicity in PC12 cells. Plant Foods for Human Nutrition (Formerly Qualitas Plantarum) 66(1):22-26.
35. de Mejia EG, et al. (2009) Bioactive components of tea: cancer, inflammation and behavior. Brain, Behavior, and Immunity 23(6):721-31.
36. Alcazar A, et al. (2007) Differentiation of green, white, black, Oolong, and Pu-erh teas according to their free amino acids content. Journal of agricultural and food chemistry 55(15):5960-65.
37. Dias TR, et al. (2017) Promising potential of dietary (poly)phenolic compounds in the prevention and treatment of diabetes mellitus. Current Medicinal Chemistry 24(4):334-52.
38. Dias TR, et al. (2013) White tea (Camellia sinensis (L.)): antioxidant properties and beneficial health effects. International Journal of Food Science, Nutrition and Dietetics 2(2):19-26.
39. Koech KR, et al. (2013) Antimicrobial, synergistic and antioxidant activities of tea polyphenols. Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education, Formatex Research Center, Badajoz:971-81.
40. Muthumani T and Kumar RS (2007) Influence of fermentation time on the development of compounds responsible for quality in black tea. Food Chemistry 101(1):98-102.
41. Leung LK, et al. (2001) Theaflavins in black tea and catechins in green tea are equally effective antioxidants. The Journal of nutrition 131(9):2248-51.
42. Stewart AJ, et al. (2005) On-line high-performance liquid chromatography analysis of the antioxidant activity of phenolic compounds in green and black tea. Molecular Nutrition and Food Research 49(1):52-60.

43. Atoui AK, et al. (2005) Tea and herbal infusions: their antioxidant activity and phenolic profile. Food chemistry 89(1):27-36.
44. Yang CS, et al. (2000) Tea and tea polyphenols in cancer prevention. The Journal of nutrition 130(2):472S-78S.
45. Wang Z-M, et al. (2011) Black and green tea consumption and the risk of coronary artery disease: a meta-analysis. The American journal of clinical nutrition 93(3):506-15.
46. Tea Association of the USA Inc (2015) Tea Fact Sheet – 2015. Available from: http://www.teausa.com/14655/tea-fact-sheet.
47. Almajano MP, et al. (2008) Antioxidant and antimicrobial activities of tea infusions. Food chemistry 108(1):55-63.
48. Pettigrew J (2004) The tea companion. Running Press.
49. Damiani E, et al. (2014) Antioxidant activity of different white teas: Comparison of hot and cold tea infusions. Journal of Food Composition and Analysis 33(1):59-66.
50. Gopal J, et al. (2016) Bactericidal activity of green tea extracts: the importance of catechin containing nano particles. Scientific reports 6:19710.
51. Ugochukwu NH, et al. (2003) The effect of Gongronema latifolium extracts on serum lipid profile and oxidative stress in hepatocytes of diabetic rats. Journal of biosciences 28(1):1-5.
52. Sun T, et al. (2014) Isolation and Bioactivities of Main Functional Components in Tea. Asian Journal of Chemistry 26(8):2191-98.
53. Khokhar S and Magnusdottir S (2002) Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom. Journal of Agricultural and Food Chemistry 50(3):565-70.
54. Lin Y-S, et al. (2003) Factors affecting the levels of tea polyphenols and caffeine in tea leaves. Journal of Agricultural and Food Chemistry 51(7):1864-73.
55. Rusak G, et al. (2008) Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and the solvent used. Food Chemistry 110(4):852-58.
56. Kuhnert N, et al. (2010) Mass spectrometric characterization of black tea thearubigins leading to an oxidative cascade hypothesis for thearubigin formation. Rapid Communications in Mass Spectrometry 24(23):3387-404.
57. Alves MG, et al. (2015) White tea consumption improves cardiac glycolytic and oxidative profile of prediabetic rats. Journal of Functional Foods 14:102-10.
58. Koutelidakis AE, et al. (2009) Green tea, white tea, and Pelargonium purpureum increase the antioxidant capacity of plasma and some organs in mice. Nutrition 25(4):453-58.
59. Nunes AR, et al. (2015) Daily consumption of white tea (Camellia sinensis (L.)) improves the cerebral cortex metabolic and oxidative profile in prediabetic Wistar rats. British Journal of Nutrition 113(05):832-42.
60. Leenen R, et al. (2000) A single dose of tea with or without milk increases plasma antioxidant activity in humans. European Journal of Clinical Nutrition 54(1):87-92.
61. Horie N, et al. (2005) Synergistic effect of green tea catechins on cell growth and apoptosis induction in gastric carcinoma cells. Biological and Pharmaceutical Bulletin 28(4):574-79.
62. Kakuda T, et al. (2000) Inhibiting effects of theanine on caffeine stimulation evaluated by EEG in the rat. Bioscience, biotechnology, and biochemistry 64(2):287-93.
63. Tenore GC, et al. (2013) In vitro hypoglycaemic and hypolipidemic potential of white tea polyphenols. Food Chemistry 141(3):2379-84.
64. Islam MS (2011) Effects of the aqueous extract of white tea (Camellia sinensis) in a streptozotocin-induced diabetes model of rats. Phytomedicine 19(1):25-31.
65. Ikeda I, et al. (1992) Tea catechins decrease micellar solubility and intestinal absorption of cholesterol in rats. Biochimica et Biophysica Acta (BBA)-lipids and lipid MetaboLism 1127(2):141-46.
66. Murase T, et al. (2002) Beneficial effects of tea catechins on diet-induced obesity: stimulation of lipid catabolism in the liver. International journal of obesity 26(11):1459-64.
67. Zheng G, et al. (2004) Anti-obesity effects of three major components of green tea, catechins, caffeine and theanine, in mice. In Vivo 18(1):55-62.
68. Söhle J, et al. (2009) White Tea extract induces lipolytic activity and inhibits adipogenesis in human subcutaneous (pre)-adipocytes. Nutrition and metabolism 6(1):20.
69. Johnson RJ, et al. (2007) Potential role of sugar (fructose) in the epidemic of hypertension, obesity and the metabolic syndrome, diabetes, kidney disease, and cardiovascular disease. The American journal of clinical nutrition 86(4):899-906.
70. Stangl V, et al. (2006) The role of tea and tea flavonoids in cardiovascular health. Molecular nutrition and food research 50(2):218-28.
71. Stoclet J-C, et al. (2004) Vascular protection by dietary polyphenols. European journal of pharmacology 500(1):299-313.
72. Tijburg LBM, et al. (1997) Tea flavonoids and cardiovascular diseases: a review. Critical Reviews in Food Science and Nutrition 37(8):771-85.
73. Yokogoshi H, et al. (1995) Reduction effect of theanine on blood pressure and brain 5-hydroxyindoles in spontaneously hypertensive rats. Bioscience, biotechnology, and biochemistry 59(4):615-18.

74. Wang X and Michaelis EK (2010) Selective neuronal vulnerability to oxidative stress in the brain. Frontiers in Aging Neuroscience 2:12.
75. Halliwell B (2006) Oxidative stress and neurodegeneration: where are we now? Journal of neurochemistry 97(6):1634-58.
76. Mandel S and Youdim MB (2004) Catechin polyphenols: neurodegeneration and neuroprotection in neurodegenerative diseases. Free Radical Biology and Medicine 37(3):304-17.
77. Kimura K, et al. (2007) L-Theanine reduces psychological and physiological stress responses. Biological psychology 74(1):39-45.
78. Valko M, et al. (2007) Free radicals and antioxidants in normal physiological functions and human disease. The international journal of biochemistry & cell biology 39(1):44-84.
79. Hajiaghaalipour F, et al. (2015) White tea (Camellia sinensis) inhibits proliferation of the colon cancer cell line, HT-29, activates caspases and protects DNA of normal cells against oxidative damage. Food Chemistry 169:401-10.
80. Santana-Rios G, et al. (2001) Potent antimutagenic activity of white tea in comparison with green tea in the Salmonella assay. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 495(1–2):61-74.
81. Mao JT, et al. (2010) White Tea Extract Induces Apoptosis in Non–Small Cell Lung Cancer Cells: the Role of Peroxisome Proliferator-Activated Receptor-γ and 15-Lipoxygenases. Cancer Prevention Research 3(9):1132-40.
82. Nazer A, et al. (2005) Combinations of food antimicrobials at low levels to inhibit the growth of Salmonella sv. Typhimurium: a synergistic effect? Food Microbiology 22(5):391-98.
83. Taguri T, et al. (2006) Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure. Biological and Pharmaceutical Bulletin 29(11):2226-35.
84. Liu S, et al. (2005) Theaflavin derivatives in black tea and catechin derivatives in green tea inhibit HIV-1 entry by targeting gp41. Biochimica et Biophysica Acta (BBA)-General Subjects 1723(1):270-81.
85. Hunt KJ, et al. (2010) Botanical extracts as anti-aging preparations for the skin. Drugs & aging 27(12):973-85.
86. Thring TSA, et al. (2011) Antioxidant and potential anti-inflammatory activity of extracts and formulations of white tea, rose, and witch hazel on primary human dermal fibroblast cells. Journal of Inflammation (London, England) 8:27.
87. Lee KO, et al. (2014) Anti-wrinkle Effects of Water Extracts of Teas in Hairless Mouse. Toxicological Research 30(4):283-89.
88. Camouse MM, et al. (2009) Topical application of green and white tea extracts provides protection from solar-simulated ultraviolet light in human skin. Experimental dermatology 18(6):522-26.
89. Saric S and Sivamani RK (2016) Polyphenols and Sunburn. International Journal of Molecular Sciences 17(9):1521.
90. Manach C, et al. (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. The American journal of clinical nutrition 81(1):230S-42S.
91. Market Research Report (2016) Dietary Supplements Market Analysis By Ingredient (Botanicals, Vitamins, Minerals, Amino Acids, Enzymes), By Product (Tablets, Capsules, Powder, Liquids, Soft Gels, Gel Caps), By Application (Additional Supplement, Medicinal Supplement, Sports Nutrition), By End-Use (Infant, Children, Adults, Pregnant Women, Old-Aged) And Segment Forecasts To 2024.
92. Abourashed EA, et al. (2016) Content Variation of Catechin Markers, Total Phenolics and Caffeine in Green Tea Dietary Supplements. Journal of dietary supplements 13(2):171-84.
93. Bettuzzi S, et al. (2006) Chemoprevention of human prostate cancer by oral administration of green tea catechins in volunteers with high-grade prostate intraepithelial neoplasia: a preliminary report from a one-year proof-of-principle study. Cancer research 66(2):1234-40.
94. Westerterp-Plantenga MS, et al. (2005) Body weight loss and weight maintenance in relation to habitual caffeine intake and green tea supplementation. Obesity 13(7):1195-204.
95. Hsu S (2005) Green tea and the skin. Journal of the American Academy of Dermatology 52(6):1049-59.

Chapter 2

Objectives

Tea is one of the most consumed beverages worldwide, mostly due to its potent antioxidant potential and beneficial health effects. However, the effects of tea in male reproductive function have been overlooked so far. Male reproduction is highly affected by oxidative stress-induced diseases, namely diabetes *mellitus* (DM). We hypothesized that tea could have a protective role on Sertoli cells (SCs) function, testicular and epididymal physiology and metabolism, as well as in sperm quality. In this research project, we aimed to unravel the effects of white tea (WTEA) on male reproductive function and its protective role against reproductive dysfunctions induced by prediabetes. Moreover, we also aimed to investigate if the effects of WTEA are due to a combined effect of all the WTEA components or to a predominant effect of one of its most bioactive components. The project was divided into three parts:

I - *In vitro* studies (chapter 3)

a) Firstly, we prepared a WTEA aqueous extract, from a commercial brand available on the Portuguese market, and determined its phytochemical profile by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. Using a primary culture of rat SCs, we evaluated the effect of our selected WTEA extract (0.5 mg/mL) in rat SCs glucose metabolism.
From the chemical characterization of the selected WTEA extract, we chose the most bioactive components: caffeine, epigallocatechin gallate (EGCG), and L-theanine, representative of the principal chemical classes found in WTEA, methylxanthines, phenolic compounds and free amino acids, respectively. This allowed us to evaluate the effects of each component individually in SCs and compare with the effects of the WTEA extract.

b) Caffeine is a widely consumed substance present in several beverages, including tea, coffee, and energetic drinks, which are popular among young individuals in reproductive age. However, caffeine safety for male reproductive function is poorly understood. As SCs are essential for a normal sperm production and hence for male fertility, we investigated the effect of caffeine (5, 50 and 500 μM) on the glycolytic and oxidative profile of cultured human SCs (hSCs) from men with conserved spermatogenesis. The used concentrations were selected based on doses reported in human plasma after low, moderate or heavy daily consumption of caffeine.

c) EGCG is the most abundant and biologically active WTEA polyphenol. Most of the tea's medicinal properties are attributed to EGCG, which has demonstrated a potent preventive activity against OS. We evaluated the effect of EGCG (5 and 50 μM) on the metabolism, mitochondrial functionality and oxidative profile of cultured hSCs from men with conserved spermatogenesis. The lowest EGCG dose represents the plasma levels attained after tea or EGCG ingestion, while the highest dose showed pharmacological relevance in previous studies. Besides, the use of these concentrations allowed the comparison with caffeine at same concentrations.

d) L-theanine is the major free amino acid present in WTEA. It showed a protecting role against oxidative damages in various cells, but its effect on testicular cells has never been investigated. We evaluated the effect of a dose of L-theanine attained by tea intake (5 µM) or a pharmacological dose (50 µM) on the metabolism, mitochondrial functionality, and oxidative profile of cultured hSCs from men with conserved spermatogenesis. The use of the same doses permitted the comparison of L-theanine results with those obtained with caffeine and EGCG.

II - *Ex vivo* studies (chapter 4)

a) Storage of spermatozoa under refrigerated conditions induces a rapid decline in viability, especially due to oxidative unbalance. On the other hand, storage at room temperature (RT) for short-term periods may be advantageous in certain situations such as the transport of sperm samples. Hence, there is a growing interest in the establishment of an optimal medium for RT sperm storage. We aimed to investigate the effect of WTEA extract (0.5 and 1 mg/mL) on the survival of rat epididymal spermatozoa at RT, for 3 days. In this study, we also used a green tea (GTEA) extract from the same manufacturer for comparative purposes.

b) Caffeine, EGCG and L-theanine are major components of WTEA and main representatives of the classes of methylxanthines, phenolic compounds and free amino acids present in this beverage. There are many studies reporting the health benefits of tea and its components. However, it is not clear if those effects are mediated by a single component or by a combined action between all tea components. Thus, we evaluated the individual and combined effects of caffeine (71 µg/mL), EGCG (82 µg/mL), and L-theanine (19 µg/mL) on rat epididymal spermatozoa survival and oxidative profile during a 3-day storage at RT. The concentrations were selected according to the concentrations of these compounds on the WTEA extract for comparative purposes.

III - *In vivo* study (chapter 5)

a) Finally, we developed a streptozotocin (STZ)-induced prediabetic rat model to characterize the *in vivo* effects of the regular ingestion of WTEA on male reproductive function on that disease model. Prediabetes has been associated with alterations in male reproductive tract, especially in testis and epididymis, resulting in reduced sperm quality. The study of this condition is extremely important to find new ways to prevent the development its progression to a more severe state and associated health complications. Herein, we aimed to determine if WTEA ingestion by prediabetic adult rats for two months could improve the metabolic alterations induced by the disease in testicular and epididymal tissues, preserving sperm quality.

Chapter 3

I - In Vitro Studies

This chapter was adapted from the following publications:

a) Martins AD, Alves MG, Bernardino RL, **Dias TR**, Silva BM, Oliveira PF. (2013) "Effect of white tea (Camellia sinensis (L.)) extract in the glycolytic profile of Sertoli cell". European Journal of Nutrition 53(6):1383-1391 (DOI:10.1007/s00394-013-0640-5).

b) **Dias TR**, Alves MG, Bernardino RL, Martins AD, Moreira AC, Silva J, Barros A, Sousa M, Silva BM, Oliveira PF. (2015) "Dose-dependent effects of caffeine in human Sertoli cells metabolism and oxidative profile". Toxicology 328:12-20 (DOI:10.1016/j.tox.2014.12.003).

c) **Dias TR**, Alves MG, Silva J, Barros A, Sousa M, Casal S, Silva BM, Oliveira PF. (2017) "Implications of epigallocatechin gallate in cultured human Sertoli cells glycolytic and oxidative profile" Toxicology In Vitro 41:214-222 (DOI:10.1016/j.tiv.2017.03.006).

d) **Dias TR**, Bernardino RL, Alves MG, Silva J, Barros A, Sousa M, Casal S, Silva BM, Oliveira PF. (2019) "L-theanine promotes cultured human Sertoli cells proliferation and modulates glucose metabolism" European Journal of Nutrition (DOI:10.1007/s00394-019-01999-2).

Effect of white tea (Camellia sinensis (L.)) extract in the glycolytic profile of Sertoli cell

Abstract

Many health benefits have been attributed to tea (*Camellia sinensis* (L.)). Tea infusions are used as dietary agents and included in food supplements. Herein, we report the effect of a white tea (WTEA) extract in Sertoli cells (SCs) metabolism. The SCs are essential for male fertility as they are responsible for the nutritional and physical support of the developing germ cells. An aqueous WTEA extract was prepared and analyzed by proton nuclear magnetic resonance (^{1}H-NMR). Rat SCs were cultured with or without the WTEA extract. Messenger ribonucleic acid (mRNA) and protein levels of glucose transporters 1 and 3 (GLUT1 and GLUT3), phosphofructokinase 1, lactate dehydrogenase (LDH) and monocarboxylate transporter 4 were determined by quantitative polymerase chain reaction and Western blot. LDH activity was also assessed, as well as metabolite production/consumption by ^{1}H-NMR. WTEA-exposed SCs presented decreased protein and mRNA levels of GLUT1 and decreased glucose uptake. However, intracellular LDH activity, lactate and alanine production increased in SCs exposed to the WTEA extract. WTEA extract altered the glycolytic profile of cultured SCs, stimulating lactate production. As lactate is used as metabolic substrate and has an anti-apoptotic effect in the developing germ cells, the supplementation of SCs with WTEA extract may be advantageous to improve male reproductive health.

Keywords: germ cells; lactate; male fertility; metabolism; Sertoli cells; white tea.

Introduction

Tea (*Camellia sinensis* (L.)) is cultivated in more than 30 countries and is the most widely consumed and popular beverage in the world, aside from water. Many health benefits have been ascribed to tea, especially to green tea (GTEA), including for several chronic diseases, such as cardiovascular diseases, cancer, and obesity [1]. Tea is rich in polyphenolic compounds that dictate its potent antioxidant potential [2]. Tea extracts are particularly rich in catechins, which are known to interact with large biomolecules such as proteins and enzymes [3]. Indeed, tea supplementation influences several metabolic pathways, showing ability to suppress insulin resistance [4] and improve insulin sensitivity [4]. Tea polyphenols were reported to inhibit intestinal glucose uptake through its antagonist action against Na^+-dependent glucose transporter [5]. Interestingly, GTEA supplementation could induce different metabolic effects during rest and exercise [6]. The plasma metabolic profile of individuals consuming a GTEA extract revealed that it enhances fat oxidation, activates the Krebs cycle, reduces amino acid catabolism and enhances glycolysis [6]. GTEA extract also increased plasma lactate concentration, which was suggested to inhibit lipolysis and limit fat oxidation rates during

exercise [6]. In human urine, following 2 days of GTEA supplementation, it was detected that GTEA catechins increase human oxidative energy metabolism and/or biosynthetic pathways [7]. Epigallocatechin gallate (EGCG), one of the most active antioxidant components of tea, showed protective effects against testicular ischemia-reperfusion injury [8]. Besides, several studies reported that tea catechins can bind and downregulate estrogen receptors [9], which are known to be expressed in testicular cells, including the Sertoli cells (SCs). SCs are the somatic testicular cells essential that form the blood-testis barrier and are key players for spermatogenesis. They are responsible for the physical and nutritional support of the developing germ cells [10]. The SCs produce lactate from several metabolic sources, especially glucose, being then exported to the intratubular compartment to be used by the developing germ cells as an energy substrate [11]. SCs have a great metabolic plasticity and are very sensitive to hormonal fluctuations [12-15] and exposure to toxicants [16]. Therefore, they are a valuable model to evaluate the possible effect of substances on male reproductive health.

White tea (WTEA) is one of the less studied types of tea. Although, it is usually reported to contain higher levels of antioxidants than green, oolong and black teas [17]. This is associated with the tea leaves collection and processing, as WTEA is produced from very young *Camellia sinensis* leaves or buds, and suffers minimal processing to prevent catechins oxidation [18]. Generally, herbal medicinal preparations are complex mixtures that contain a wide variety of compounds. It is expected that the combined action of all the compounds is responsible for the beneficial effects of herbal preparations [19]. However, the worldwide popularity of tea and the increasing scientific interest in its health benefits, has led to the widespread use of tea as a natural dietary agent and the inclusion of tea extracts in oral food supplements without investigating its effects in several physiological functions, such as male reproductive potential. Herein, we evaluated the effect of a WTEA aqueous extract in cultured rat SCs function.

Materials and methods

Chemicals

Deuterium oxide (D_2O; 99.9%) was purchased from Cambridge Isotope Laboratories Inc. (Cambridge, MA, USA). Taq deoxyribonucleic acid (DNA) polymerase was purchased from Fermentas Life Sciences (Ontario, Canada). Random primers, polyclonal antibodies and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) were purchased from Invitrogen (Carlsbad, CA, USA). Deoxynucleotide Triphosphates (dNTPs) were purchased from GE Healthcare (Buckinghamshire, UK) and Fetal Bovine Serum (FBS) was obtained from Biochrom AG (Berlin, Germany). Maxima SYBR Green/Fluorescein quantitative polymerase chain reaction (qPCR) Master Mix was purchased from Thermo Scientific (California, USA). All the other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specifically stated.

Animals

Ten 20-day old male Wistar rats (*Rattus norvegicus*) were housed under a 12 h light-12 h darkness cycle and constant room temperature (RT; 20 ± 2°C) in accredited animal facilities (Faculty of Health Sciences, University of Beira Interior, Portugal). Animals were maintained with food and water *ad libitum*. Accommodation, maintenance and animal handling were performed according to the "Guide for the Care and Use of Laboratory Animals"; available by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the rules for the care and handling of laboratory animals (Directive 2010/63/EU). We used 20-day old rats because at this stage SCs have a high capacity to divide and proliferate [20]. Besides, SCs primary cultures attained from 20-day old rats remain responsive to stimuli such as hormones and pollutants and have less contamination from other testicular cells [11, 12, 16].

Sertoli cell culture

Animals were euthanized by cervical dislocation and testes immediately excised in aseptic conditions and washed twice with 20 mL of cold Hanks' Balanced Salt solution (HBSS) containing 10.000 U/mL of penicillin, 10 mg/mL of streptomycin and 25 µg/ml amphotericin B (pH 7.4). SCs were isolated and cultured (in phenol-red free media) using a previously described method by Rato and collaborators [12]. Briefly, tissue from decapsulated testes was placed in glycine medium (HBSS plus 1 M glycine, 2 mM ethylenediamine tetraacetic acid (EDTA), 0.002% (w/v) soybean trypsin inhibitor; pH 7.2). The tubular pellet was digested with Collagenase type I and DNase at room temperature. The cellular suspension was then collected by centrifugation, washed in HBSS and resuspended in SCs culture medium (1:1 mixture of DMEM:F12, pH 7.2–7.4) supplemented with 15 mM hydroxyethyl piperazineethanesulfonic acid (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. The SCs suspension was then forced through a 20G needle to disaggregate possible clusters, placed on culture flasks (Cell+; Sarstedt), and incubated at 33°C in an atmosphere of 5% CO_2, 95% O_2 in SCs culture medium. SCs culture purity was determined by the immunoperoxidase detection of the specific marker Vimentin (Dako, Glostrup, Denmark, M072501), using standard methods [12, 16, 21]. Cultures were examined through an inverted microscope and selected if cells contaminants were below 5%.

White tea extract preparation

WTEA was purchased on the Portuguese market. Samples (n = 5) were subjected to infusion following the manufacturer's instructions (1 g/100 mL) at 100°C for 3 minutes. The resulting infusion was then filtered with a sterile filter of 0.2 µm cellulose acetate (VWR, Pennsylvania, USA), and freeze-dried in a ScanVac CoolSafe Freeze Dryer™ (Labogene, Lynge, Denmark). The aqueous extraction yield was 25% and the lyophilized extract was kept in a desiccator, in the dark, until analysis.

Experimental groups

SCs could grow until reach 90–95% confluence, and then were washed thoroughly and the medium replaced by serum and phenol-red free media (DMEM:F12, 1:1, with insulin-transferrin-sodium selenite (ITS) supplement, pH 7.4). SCs were treated or not with 0.5 mg/mL of WTEA extract. After a 50-h incubation, SCs were detached with a trypsin–EDTA solution and collected using standard methods. A viability test was performed using the Trypan Blue Exclusion Test. Viability averaged 85–95%, always with values higher than 85%. At the end of the treatment, the total number of cells per flask was determined with a Neubauer chamber, extracellular media was collected for proton nuclear magnetic resonance (^{1}H-NMR) and cells were collected for ribonucleic acid (RNA) or protein extraction.

Extraction of total RNA and synthesis of complementary DNA

Extraction of total RNA of cells was performed using the E.Z.N.A.® Total RNA Kit I (Omega bio-tek, Norcross, USA) following the manufacturer's instructions. RNA concentration and absorbance ratios (A260/A280) were determined by spectrophotometry (Nanophotometer™, Implen, Germany). The RNA obtained from each sample was reversely transcribed using a mixture containing 0.5 mM of each dNTP, 250 ng of random hexamer primers, 1 µg of RNA and sterile H_2O up to a final volume of 17 µL. The mixture was initially incubated 5 minutes at 65°C. Then, 200 U of M-MLV RT and 2 µL of Reaction Buffer were added and incubated sequentially at 25°C for 10 minutes, 37°C for 50 minutes and 70°C for 15 minutes.

Quantitative polymerase chain reaction

qPCR was performed to analyze monocarboxylate transporter 4 (MCT4), glucose transporter 1 (GLUT1), glucose transporter 3 (GLUT3), phosphofructokinase 1 (PFK1) and lactate dehydrogenase (LDH) messenger RNA (mRNA) expression. Specific primers were designed for the amplification of the target and housekeeping transcripts (Table 3.1). qPCR was carried out using an iQ5 system (Bio-Rad, Hercules, USA) and efficiency of the amplification was determined for all primer sets using serial dilutions of complementary DNA (cDNA). qPCR conditions were optimized, and the specificity of the amplicons was determined by melting curve analysis. qPCR amplifications used 1 µg of synthesized cDNA in a 20 µL reaction containing: 10 µL Maxima SYBR Green/Fluorescein qPCR Master Mix and 0.3 µM of sense and antisense primers for each gene. Amplification conditions comprised an initial denaturation of 5 minutes at 95°C, followed by cycles of: i) denaturation, 95°C for 10 s; ii) annealing, during 30 s (Table 3.1); and iii) extension, 72°C for 10 s. β-2-microglobulin (B2M) transcript levels were used to normalize the mRNA expression of MCT4, GLUT1, GLUT3, PFK1 and LDH. Fold variation of the expression of target genes was calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta Ct}$.

Table 3.1 Oligonucleotides and cycling conditions for amplification of glucose transporters (GLUT1 and GLUT3), phosphofructokinase 1 (PFK1), lactate dehydrogenase (LDH), monocarboxylate transporter 4 (MCT4) and ß-2-microglobulin (B2M).

Gene	Primer Sequence (5'-3')	AT (°C)	Amplicon size (base pair)	No. of cycles
GLUT1	Sense: TCCATTCTCCGTTTCACAGC Antisense: CCGGTGTTATAGCCGAACTG	55	145	40
GLUT3	Sense: GCTCTGGTCGTTATGTGTGG Antisense: TCAACCGACTCCGCTATCTT	62	171	35
PFK1	Sense: TGATTGGCTGTTCATCCCTG Antisense: GAGATAGGCTTTCCATGCCG	50	55	35
LDH	Sense: CGTCGTCCCCCATCGTGCAC Antisense: GGGCCCCCGCGGTGATAATG	60	345	35
MCT4	Sense: ACACTTAGGAGACAACAC Antisense: GGCAATATAGGAGACTGG	47	132	37
ß2M	Sense: ATGAGTATGCCTGCCGTGTG Antisense: CAAACCTCCATGATGCTGCTTAC	58	92	30

Abbreviations: AT, annealing temperature.

Western blot

Western blot procedure was performed as previously described by Alves and collaborators [22]. The membranes were incubated overnight at 4°C with rabbit anti-GLUT1 (1:300, Millipore, Temecula, USA, CBL242), or rabbit anti-GLUT3 (1:500, Abcam, Cambridge, MA, ab41525), or rabbit anti-PFK1 (1:400, Santa Cruz Biotechnology Heidelberg, Germany, Sc 67028), or rabbit anti-MCT4 (1:1000, Santa Cruz Biotechnology Heidelberg, Germany, Sc 50329), or rabbit anti-LDH (1:10000, Abcam, Cambridge, MA, ab52488). Mouse anti-α-tubulin was used as protein loading control (1:5000, Sigma, Roedermark, Germany, T 9026). The immune-reactive proteins were detected separately with goat anti-rabbit IgG-alkaline phosphatase (AP) (1:5000, Santa Cruz Biotechnology Heidelberg, Germany, Sc 2007) or goat anti-mouse IgG-AP (1:5000, Santa Cruz Biotechnology Heidelberg, Germany, Sc 2008). Membranes were reacted with ECF detection system (GE, Healthcare, Weßling, Germany) and read with the Bio-Rad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK). The Quantity One Software (Bio-Rad, Hemel Hempstead, UK) was used to obtain band densities following standard procedures. The obtained band density was divided by the respective α-tubulin band density and then presented in fold variation to control.

Lactate dehydrogenase activity assay

LDH activity was determined using a commercial assay kit (Promega, Madison, WI, USA) and following the manufacturer's instructions. Briefly, LDH enzymatic activity was calculated by measuring the shift on the absorbance (492 nm) of protein samples that resulted from the conversion of a tetrazolium salt (INT or 3-(4-Iodophenyl)-2-(4-nitrophenyl)-5-phenyl-2H-tetrazol-3-ium chloride) into a red formazan product. The amount of formazan formed is directly proportional to the activity of LDH on the samples. The method was calibrated with the LDH positive control included in the assay kit. The attained activities were calculated using the molar absorptivity of formazan and expressed in nmol/min/mg protein.

Proton nuclear magnetic resonance spectroscopy

^{1}H-NMR spectra of the extracellular media and the WTEA extract were acquired at 14.1 T, 25°C, using a Bruker Avance 600 MHz spectrometer equipped with a 5-mm QXI probe with a z-gradient (Bruker Biospin, Karlsruhe, Germany) using standard methods [16, 23]. Sodium fumarate (final concentration of 2 mM) was used as internal reference (singlet, 6.50 ppm) to quantify the metabolites in solution (multiplet, δ, ppm): L-theanine (triplet, 1.08); lactate (doublet, 1.33); alanine (doublet, 1.45); acetate (singlet, 1.9); EGCG (doublet, 2.7) caffeine (singlet, 3.29); H1-α glucose (doublet, 5.22); sucrose (doublet, 5.4); fumarate (singlet, 6.5); epigallocatechin (EGC) (singlet, 6.6); epicatechin (EC) (singlet, 7.0). The relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro NMR spectral analysis program (Acorn NMR, Inc, Fremont, CA, USA).

Statistical analysis

The statistical significance among the experimental groups was assessed by two-tailed Mann-Whitney test for independent samples, using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Results were expressed as mean ± SEM (n = 5 for each condition). $P < 0.05$ was considered significant.

Results

WTEA extract is rich in catechins and caffeine

Tea extracts are complex mixtures of phytochemicals and may vary according to the extraction methods (solvents, time of extraction, temperatures), type of tea (white, green, oolong, or black), and the geographical origin of the tea leaves. Our WTEA aqueous extract was essentially composed by EGCG (8%), caffeine (7%), sucrose (6%), EGC (5%) and L-theanine (2%). It was also possible to identify glucose (0.6%), EC (0.5%), lactate (0.04%) and alanine (0.07%) (Table 3.2).

Table 3.2 Phytochemical profile of white tea (WTEA) extract in percentage of weight determined for each compound relative to the total weight of WTEA extract.

Compound	Content ($\%_{(w/w)}$ of WTEA extract)
Glucose	0.6 ± 0.1
Sucrose	6 ± 4
Lactate	0.040 ± 0.001
Alanine	0.07 ± 0.01
Caffeine	7 ± 1
L-Theanine	2 ± 0.2
EC	0.5 ± 0.1
EGC	5 ± 1
EGCG	8 ± 1

Abbreviations: EC, epicatechin; EGC, epigallocatechin; EGCG, epigallocatechin gallate.

Glucose consumption decreased in rat SCs exposed to WTEA extract

In normal conditions, glucose is the preferred substrate of SCs for lactate production, which is crucial for the progression of spermatogenesis. Our results showed that the extracellular glucose uptake was decreased in SCs exposed to the WTEA extract. SCs from the control group consumed 83 ± 16 pmol/cell, while those exposed to the WTEA extract consumed 18 ± 3 pmol/cell (Figure 3.1A). The glucose transport to SCs is mediated by GLUT1 and GLUT3 [12, 24], thus we determined mRNA and protein levels of both transporters. No differences were observed in GLUT3 mRNA or protein levels between the experimental groups (Figure 3.2A, B), However, GLUT1 mRNA levels were lower in SCs exposed to WTEA extract (0.52 ± 0.05-fold variation to control) (Figure 3.2A), as well as GLUT1 protein levels (0.19 ± 0.03-fold variation to control) (Figure 3.2B). A rate-limiting step of glycolysis is the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate by PFK1. Although the SCs exposed to the WTEA extract did not show altered PFK1 mRNA levels, PFK1 protein levels were decreased (0.34 ± 0.05-fold variation to control) in WTEA-exposed SCs (Figure 3.2B).

WTEA extract stimulates LDH activity and lactate production by SCs

One of the most important SCs functions is lactate production, as it is used by germ cells for energy production [10]. The intracellular LDH levels and activity are crucial for lactate production by SCs. Therefore, we determined LDH mRNA and protein levels, as well as LDH activity. WTEA-exposed SCs showed a decrease in LDH mRNA levels (0.10 ± 0.01-fold variation to control) relative to control (Figure 3.2A), but no differences were observed in LDH protein levels (Figure 3.2B). Nevertheless, LDH activity was stimulated by WTEA extract exposure. The LDH activity increased from 34 ± 3 nmol/min/mg protein in the control group to 56 ± 6 nmol/min/mg protein in SCs exposed to the WTEA extract (Figure 3.3). This resulted in the increase of lactate production in SCs exposed to the WTEA extract (39 ± 6 pmol/cell), when compared to the control group (Figure 3.1B).

MCT4 plays a key role in lactate export by SCs to the intratubular fluid, so lactate can be consumed by germ cells [12]. WTEA extract induced a decrease in MCT4 mRNA levels (0.56 ± 0.07-fold variation to control) (Figure 3.2A), but there were no differences in MCT4 protein levels between the experimental groups (Figure 3.2B).

Acetate production was not altered while alanine production increased in SCs exposed to WTEA extract

SCs can also produce high amounts of acetate and alanine [13]. Although the exact role for this acetate production and export by SCs remains largely unknown, it was suggested that acetate may be essential to maintain a high rate of lipid synthesis in the developing germ cells [13]. Acetate production by SCs was not altered by the exposure to the WTEA extract (Figure 3.1D). Though, alanine production increased in SCs exposed to WTEA (4.0 ± 0.3 pmol/cell) relative to the control group (2.3 ± 0.7 pmol/cell) (Figure 3.1C).

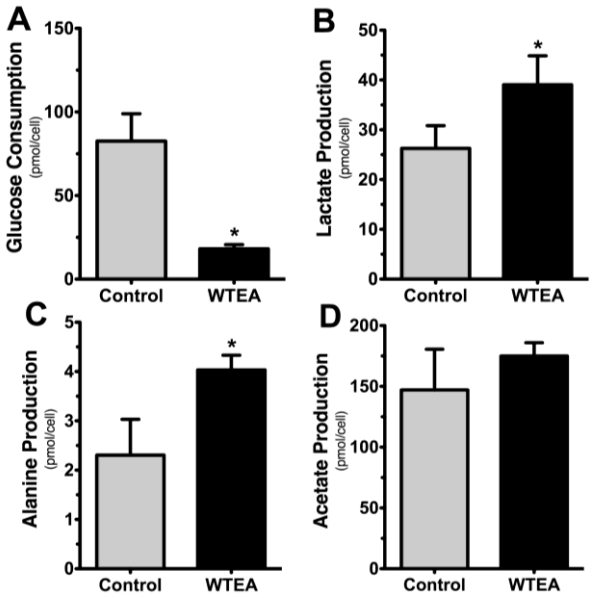


Figure 3.1 Effect of white tea (WTEA) extract in metabolites consumption/production by cultured rat Sertoli cells (SCs). The figure shows pooled data of independent experiments, indicating glucose consumption (Panel A) and the production of lactate (Panel B), alanine (Panel C), and acetate (Panel D) by cultured SCs (pmol/cell). Results are expressed as mean ± SEM (n = 5 for each condition). Significantly different results ($p < 0.05$) are indicated as: * relative to control.

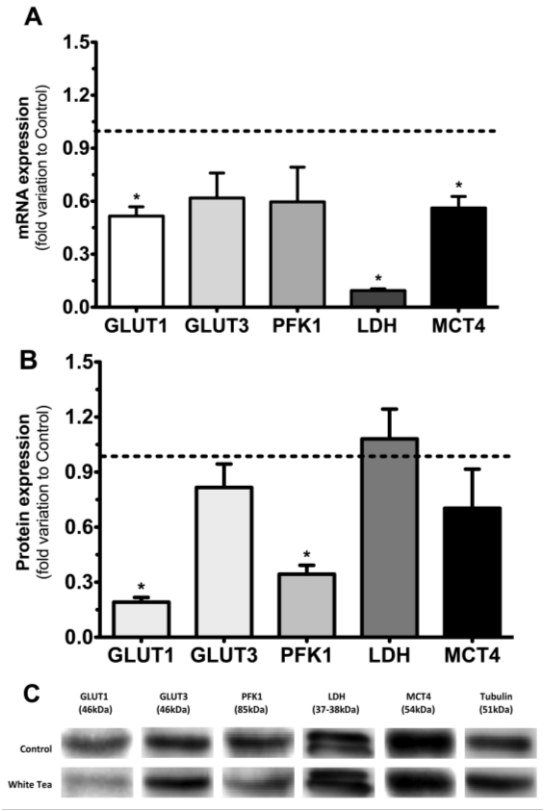


Figure 3.2 Effect of white tea (WTEA) extract in mRNA (Panel A) and protein levels (Panel B) of glucose transporter 1 (GLUT1), glucose transporter 3 (GLUT3), phosphofructokinase 1 (PFK1), lactate dehydrogenase (LDH) and monocarboxylate transporter 4 (MCT4). Representative blots are also presented (Panel C). The figure shows pooled data of independent experiments, indicating the fold variation of mRNA and protein levels observed in rat SCs cultured supplemented with a WTEA extract relative to the control group (dashed line). Results are expressed as mean ± SEM (n = 5 for each condition). Significantly different results ($p < 0.05$) are indicated as: * relative to control.

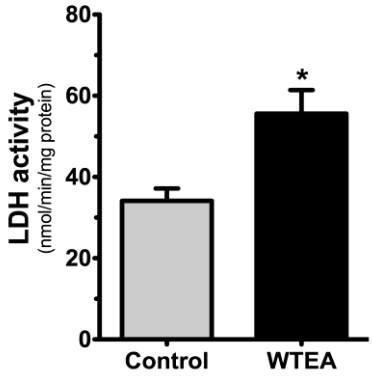


Figure 3.3 Effect of white tea (WTEA) extract in cultured rat Sertoli cells (SCs) intracellular lactate dehydrogenase (LDH) activity. The figure shows pooled data of independent experiments, indicating LDH activity in nmol/min/mg protein. Results are expressed as mean ± SEM (n = 5 for each condition). Significantly different results ($p < 0.05$) are indicated as: * relative to control.

Discussion

Several tea-based food supplements enter the global market every year. Although the beneficial health effects of tea have been widely debated [25], the effect of tea extracts in male reproductive function, namely in testicular cells functions remains scarce. We have selected a WTEA extract because it is still poorly studied, it is reach in antioxidant compounds, and tea extracts are more stable than pure catechins alone [26]. Moreover, the overall preventive effect of GTEA observed *in vivo* is thought to require the combined actions of several components rather than a single compound [27]. Therefore, we evaluated the effects of an aqueous WTEA extract in rat cultured SCs function. Our focus was SCs metabolism as it is essential for the normal progression of spermatogenesis [11]. Besides, SCs are known to be sensitive to toxicants [16] and a target for reproductive hazards [28].

Glucose consumption by SCs was reduced by the exposure to the WTEA extract. The transport of glucose across cells membrane is a rate-limiting step for glucose metabolism [29]. In SCs, the extracellular glucose uptake is mainly achieved by passive transport through GLUT1 and GLUT3 [12, 15, 24]. Thus, although GLUT3 mRNA and protein levels remained unchanged, GLUT1 mRNA and protein levels decreased in WTEA-exposed SCs. Interestingly, in insulin- [14] and glucose-deprivation [30] conditions, it was observed an increase in GLUT1 expression in SCs as an adaptative response to ensure the correct lactate production. However, SCs exposed to the WTEA extract showed a lower glucose consumption by decreasing GLUT1 levels, evidencing that WTEA action on GLUTs is selective and not compensated by GLUT3. Others have also reported that tea polyphenols inhibit intestinal glucose uptake though inhibition of glucose transporters in a competitive manner [5, 31]. It was previously demonstrated that GTEA extract can inhibit intestinal lipases [32] and tea catechins can inhibit enzymes, such as lactase, involved in carbohydrate digestion [33]. Besides, the main polyphenol present in WTEA extract, EGCG, was reported to decrease the expression of genes related to gluconeogenesis in liver [34]. Thus, the inhibition of GLUT1 and glucose uptake in rat SCs induced by our WTEA extract

is concomitant with previous cellular and molecular mechanisms reported for GTEA extracts and tea catechins.

After glucose enters the SCs, it is expected to undergo glycolysis. One of the rate-limiting steps of glucose metabolism is the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate by PFK1, finally resulting in pyruvate production. As glucose consumption was reduced, the protein levels of PFK1 were also decreased in WTEA-exposed SCs, evidencing that glucose metabolization to pyruvate is inhibited in SCs after exposure to the WTEA extract.

The SC is known for its high metabolic plasticity. It was reported that SCs cultured in unfavorable conditions, such as glucose [30] or insulin deprivation [14], show metabolic alterations that allow them to ensure an adequate lactate concentration in the microenvironment where germ cells develop. Our results demonstrated that although glucose uptake by SCs decreased after exposure to WTEA, lactate production was higher. This is concomitant with the increase in LDH activity in SCs exposed to the WTEA extract. Increased LDH activity is usually associated with an increased glycolytic flux [35]. It was previously reported that GTEA extract can also enhance glycolytic enzymes and control the glucose metabolism in the liver tissues of diabetic rats [36]. The health benefits of tea consumption are usually attributed to the high polyphenolic content. The mechanisms by which tea extracts mediate the glycolytic profile of cultured cells remains unknown. Nevertheless, our results provide evidence for the stimulation of lactate production by SCs through exposure to a WTEA extract. Due to their metabolic plasticity [12, 37, 38], SCs can metabolize other substrates such as palmitate and ketone bodies, as well as glutamine, alanine, leucine, glycine, and valine [39, 40]. They can also produce adenosine triphosphate (ATP) from ß-oxidation and through recycled lipids from apoptotic spermatogenic cells [41]. Finally, the presence of glycogen and glycogen phosphorylase activity was also identified in SCs [42]. Although the contribution of glycogen to the overall SCs metabolism remains obscure, it was suggested that, under hormonal stimulation, glycogen metabolism may be a reliable source for lactate production by SCs [12, 15]. SCs prefer glycolysis over Krebs cycle to assure the appropriate lactate production for the developing germ cells. Thus, it is possible that the exposure to the WTEA extract can stimulate the use of alternative substrates to increase lactate production. Moreover, the intratesticular injection of lactate is known to improve the spermatogenesis *in vivo* [43]. Besides, germ cells apoptosis was reported to be inhibited by lactate in a dose-dependent way [44]. Thus, the increase in lactate production induced by the exposure to a WTEA extract and the associated increase in MCT4 mRNA levels may be a good strategy to improve the male reproductive potential. Further studies focused on the metabolic cooperation between SCs and germ cells will be needed to understand the possible protective effects of this increase in lactate production by SCs to germ cells differentiation and survival.

The antioxidant properties of tea extracts are well known and usually associated to its high polyphenol content. Our results showed that SCs exposed to the WTEA extract increased not

only lactate, but also alanine production. This is crucial to maintain the lactate/alanine ratio. This ratio reflects the cellular redox state [45], as the conversion of pyruvate or its conversion into alanine is coupled with re-oxidation of nicotinamide adenine dinucleotide reduced (NADH) into nicotinamide adenine dinucleotide oxidized (NAD^+). Our results suggest that WTEA maintains the cellular redox homeostasis. The increase in alanine production counteracts the deleterious oxidative effect induced by the increased lactate production. Further studies will be needed to fully disclose this mechanism. Noteworthy, our data point towards a potential role of WTEA extract or any of its components, in the regulation of translational control and protein half-life, particularly concerning the disparate results in some enzymes/transporters for mRNA versus protein abundance. We have recently reported that these two processes (mRNA and protein expression for these enzymes) may be regulated on a different timeframe in SCs [38]. Moreover, the main WTEA catechin EGCG, induced a downregulation of the expression of some genes such as the androgen receptor in LNCaP prostate cancer cells [46]. In human colorectal cancer cells, EGCG suppressed the expression of proteins related with cell proliferation, both post-transcriptionally and post-translationally, [47]. Thus, it is possible that some WTEA extract phytochemicals, such as EGCG, regulate the gene or protein expression of the glycolytic enzymes and transporters analyzed in SCs at different timeframes.

The worldwide popularity of tea consumption and its health interest has led to the inclusion of tea extracts in food and nutritional supplements, but few studies have been focused on the effects of tea extracts in male reproductive health. The use of a WTEA extract permits the study of a higher concentration of phytochemicals relative to that achieved by tea infusion ingestion, allowing us to infer about its safety and possible medicinal use. Herein, we reported that exposure of SCs to a WTEA aqueous extract induced two major changes in the glycolytic profile of these cells: i) decreased GLUT1 levels and glucose uptake; ii) stimulated LDH activity and lactate production. These alterations in SCs metabolism may be crucial to improve male reproductive health in certain conditions as lactate is essential for germ cells survival.

References

1. Cabrera C, et al. (2006) Beneficial effects of green tea--a review. Journal of the American College of Nutrition 25(2):79-99.
2. Dufresne CJ and Farnworth ER (2001) A review of latest research findings on the health promotion properties of tea. The Journal of Nutritional Biochemistry 12(7):404-21.
3. Higdon JV and Frei B (2003) Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. Critical Reviews in Food Science and Nutrition 43(1):89-143.
4. Hininger-Favier I, et al. (2009) Green tea extract decreases oxidative stress and improves insulin sensitivity in an animal model of insulin resistance, the fructose-fed rat. Journal of the American College of Nutrition 28(4):355-61.
5. Kobayashi Y, et al. (2000) Green tea polyphenols inhibit the sodium-dependent glucose transporter of intestinal epithelial cells by a competitive mechanism. Journal of Agricultural and Food Chemistry 48(11):5618-23.
6. Hodgson AB, et al. (2013) Metabolic response to green tea extract during rest and moderate-intensity exercise. The Journal of Nutritional Biochemistry 24(1):325-34.
7. Van Dorsten FA, et al. (2006) Metabonomics approach to determine metabolic differences between green tea and black tea consumption. Journal of Agricultural and Food Chemistry 54(18):6929-38.

8. Sugiyama A, et al. (2012) Beneficial effects of (-)-epigallocatechin gallate on ischemia-reperfusion testicular injury in rats. Journal of Pediatric Surgery 47(7):1427-32.
9. Goodin MG, et al. (2002) Estrogen receptor-mediated actions of polyphenolic catechins in vivo and in vitro. Toxicological Sciences 69(2):354-61.
10. Rato L, et al. (2012) Metabolic regulation is important for spermatogenesis. Nat Rev Urol 9(6):330-8.
11. Alves MG, et al. (2013) Hormonal control of Sertoli cell metabolism regulates spermatogenesis. Cellular and Molecular Life Sciences 70(5):777-93.
12. Rato L, et al. (2012) Metabolic modulation induced by oestradiol and DHT in immature rat Sertoli cells cultured in vitro. Bioscience Reports 32(1):61-9.
13. Alves MG, et al. (2012) In vitro cultured human Sertoli cells secrete high amounts of acetate that is stimulated by 17beta-estradiol and suppressed by insulin deprivation. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1823(8):1389-94.
14. Oliveira PF, et al. (2012) Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells. Biochimica et Biophysica Acta (BBA) - General Subjects 1820(2):84-9.
15. Oliveira PF, et al. (2011) Influence of 5alpha-dihydrotestosterone and 17beta-estradiol on human Sertoli cells metabolism. International Journal of Andrology 34(6 Pt 2):e612-20.
16. Alves MG, et al. (2013) Exposure to 2,4-dichlorophenoxyacetic acid alters glucose metabolism in immature rat Sertoli cells. Reproductive Toxicology 38C:81-88.
17. Sharangi A (2009) Medicinal and therapeutic potentialities of tea (Camellia sinensis L.)–A review. Food research international 42(5):529-35.
18. Rusak G, et al. (2008) Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and the solvent used. Food Chemistry 110(4):852-58.
19. Loew D and Kaszkin M (2002) Approaching the problem of bioequivalence of herbal medicinal products. Phytotherapy Research 16(8):705-11.
20. Zivkovic D and Hadziselimovic F (2009) Development of Sertoli cells during mini-puberty in normal and cryptorchid testes. Urologia Internationalis 82(1):89-91.
21. Simoes VL, et al. (2013) Regulation of apoptotic signaling pathways by 5alpha-dihydrotestosterone and 17beta-estradiol in immature rat Sertoli cells. Journal of Steroid Biochemistry and Molecular Biology 135C:15-23.
22. Alves MG, et al. (2011) Anti-apoptotic protection afforded by cardioplegic celsior and histidine buffer solutions to hearts subjected to ischemia and ischemia/reperfusion. Journal of Cellular Biochemistry 112(12):3872-81.
23. Alves MG, et al. (2011) Substrate selection in hearts subjected to ischemia/reperfusion: role of cardioplegic solutions and gender. NMR in Biomedicine 24(9):1029-37.
24. Galardo MN, et al. (2008) Regulation of expression of Sertoli cell glucose transporters 1 and 3 by FSH, IL1 beta, and bFGF at two different time-points in pubertal development. Cell and Tissue Research 334(2):295-304.
25. Dias TR, et al. (2013) White Tea (Camellia Sinensis (L.)): Antioxidant Properties and Beneficial Health Effects. International Journal of Food Science, Nutrition and Dietetics 2(2):19-26.
26. Kaszkin M, et al. (2004) Unravelling green tea's mechanisms of action: more than meets the eye. Molecular Pharmacology 65(1):15-7.
27. Williams SN, et al. (2000) Comparative studies on the effects of green tea extracts and individual tea catechins on human CYP1A gene expression. Chemico-Biological Interactions 128(3):211-29.
28. Monsees TK, et al. (2000) Sertoli cells as a target for reproductive hazards. Andrologia 32(4-5):239-46.
29. Angulo C, et al. (1998) Hexose transporter expression and function in mammalian spermatozoa: cellular localization and transport of hexoses and vitamin C. Journal of Cellular Biochemistry 71(2):189-203.
30. Riera MF, et al. (2009) Molecular mechanisms involved in Sertoli cell adaptation to glucose deprivation. Am J Physiol Endocrinol Metab 297(4):E907-14.
31. Shimizu M, et al. (2000) Regulation of intestinal glucose transport by tea catechins. Biofactors 13(1-4):61-5.
32. Juhel C, et al. (2000) Green tea extract (AR25) inhibits lipolysis of triglycerides in gastric and duodenal medium in vitro. J Nutr Biochem 11(1):45-51.
33. Naz S, et al. (2011) Epigallocatechin-3-gallate inhibits lactase but is alleviated by salivary proline-rich proteins. Journal of Agricultural and Food Chemistry 59(6):2734-8.
34. Waltner-Law ME, et al. (2002) Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production. Journal of Biological Chemistry 277(38):34933-40.
35. Kim JW and Dang CV (2005) Multifaceted roles of glycolytic enzymes. Trends in Biochemical Sciences 30(3):142-50.
36. Sundaram R, et al. (2013) Modulatory effect of green tea extract on hepatic key enzymes of glucose metabolism in streptozotocin and high fat diet induced diabetic rats. Phytomedicine 20(7):577-84.

37. Alves MG, et al. (2013) Molecular mechanisms beyond glucose transport in diabetes-related male infertility. Biochimica et Biophysica Acta 1832(5):626-35.
38. Martins AD, et al. (2013) Control of Sertoli cell metabolism by sex steroid hormones is mediated through modulation in glycolysis-related transporters and enzymes. Cell and Tissue Research 354(3):861-8.
39. Jutte NH, et al. (1985) Metabolism of palmitate in cultured rat Sertoli cells. J Reprod Fertil 73(2):497-503.
40. Kaiser GR, et al. (2005) Metabolism of amino acids by cultured rat Sertoli cells. Metabolism 54(4):515-21.
41. Xiong W, et al. (2009) Apoptotic spermatogenic cells can be energy sources for Sertoli cells. Reproduction 137(3):469-79.
42. Slaughter GR and Means AR (1983) Follicle-stimulating hormone activation of glycogen phosphorylase in the Sertoli cell-enriched rat testis. Endocrinology 113(4):1476-85.
43. Courtens JL and Ploen L (1999) Improvement of spermatogenesis in adult cryptorchid rat testis by intratesticular infusion of lactate. Biology of Reproduction 61(1):154-61.
44. Erkkila K, et al. (2002) Lactate inhibits germ cell apoptosis in the human testis. Molecular Human Reproduction 8(2):109-17.
45. O'Donnell JM, et al. (2004) Limited transfer of cytosolic NADH into mitochondria at high cardiac workload. American Journal of Physiology: Heart and Circulatory Physiology 286(6):H2237-42.
46. Ren F, et al. (2000) Tea polyphenols down-regulate the expression of the androgen receptor in LNCaP prostate cancer cells. Oncogene 19(15):1924-32.
47. Sukhthankar M, et al. (2010) (-)-Epigallocatechin-3-gallate (EGCG) post-transcriptionally and post-translationally suppresses the cell proliferative protein TROP2 in human colorectal cancer cells. Anticancer Research 30(7):2497-503.

Dose-dependent effects of caffeine in human Sertoli cells metabolism and oxidative profile

Abstract

Caffeine is a widely consumed substance present in several beverages. There is an increasing consumption of energetic drinks, rich in caffeine, among young individuals in reproductive age. Caffeine has been described as a modulator of cellular metabolism. Hence, we hypothesized that it may alter human Sertoli cells (hSCs) metabolism and oxidative profile, which are essential for spermatogenesis. For that purpose, hSCs were cultured with increasing doses of caffeine (5, 50, 500 µM). Caffeine at the lowest concentrations (5 and 50 µM) stimulated lactate production, but only hSCs exposed to 50 µM showed increased expression of glucose transporters (GLUTs). At the highest concentration (500 µM), caffeine stimulated lactate dehydrogenase activity to sustain lactate production. Notably, the antioxidant capacity of hSCs decreased in a dose-dependent manner and hSCs exposed to 500 µM caffeine presented a pro-oxidant potential, with a concurrent increase of protein oxidative damage. Hence, moderate consumption of caffeine appears to be safe to male reproductive health, as it stimulates lactate production by hSCs, which can promote germ cells survival. Nevertheless, caution should be taken by heavy consumers of energetic beverages and foodstuffs supplemented with caffeine to avoid deleterious effects in hSCs functioning and spermatogenesis.

Keywords: caffeine; cell metabolism; lactate; male fertility; Sertoli cell; spermatogenesis.

Introduction

Caffeine is one of the most widely consumed psychoactive substances and its popularity has been attributed to its stimulant properties. It is structurally known as 1,3,7-trimethylxanthine, naturally present in over 60 plant species, but it can also be artificially manufactured [1]. The main sources of dietary caffeine are tea leaves (*Camelia sinensis*) and roasted coffee beans (*Coffea Arabica* and *Coffea robusta*), with each contributing about equally to total caffeine intake (about 240 mg/adult/day) [2]. It is estimated that tea contains 1.4-3.4 times less caffeine than coffee [3], but the total caffeine content depends on the particular leaf or bean and on how the beverage is prepared [4]. Moreover, in several countries, tea is consumed in higher doses than coffee [5]. A daily consumption of 240-300 mg of caffeine correspond to an ingestion of 3–7 mg caffeine/kg of body weight in adults [6]. Blanchard and Sawers [7] demonstrated that an oral administration of 5 mg caffeine/kg results in a plasma concentration of 10 µg/mL (50 µM). Another study reported that the intake of 300 mg of pure caffeine resulted also in a plasmatic concentration of approximately 50 mM [8]. The caffeine molecule is easily absorbed by humans, having approximately 100% of bioavailability when taken by oral route and reaching a peak in the blood within 15–45 min after its consumption [9]. After being

absorbed, caffeine is distributed to various tissues and broken down to metabolites with variable pharmacological actions [6]. While the moderate consumption of caffeine is usually seen as a relatively good practice, there are several studies indicating that when taken in excessive amounts it may lead to various deleterious health effects [9]. Of particular concern is the increasing consumption of energy drinks that are rich in caffeine and very popular among young people (for review [10]). Besides, caffeine can also be found in products containing cocoa or chocolate, as well as in several medications and dietary supplements [1, 11]. The major health problems concerning caffeine and human disease include coronary heart disease, reproductive disorders, and psychiatric disturbances [12].

The most important mechanism of action of caffeine appears to be the antagonism of adenosine receptors [13]. As caffeine molecular structure is similar to adenosine, with both compounds having a double bond ring structure, caffeine has the potential to occupy adenosine receptor sites [14]. Adenosine and its antagonists have long been suggested to influence the male reproductive system [15]. Several studies have demonstrated the presence of adenosine receptors in Sertoli cells (SCs) [16, 17] and showed that these cells can be modulated by adenosine and its analogues [18]. The somatic SCs are responsible for the functional development of the testis and hence for the expression of the male phenotype [19, 20]. They also actively metabolize several substrates, especially glucose, to ensure lactate supply to the developing germ cells [21]. Thus, the overall metabolic functioning of SCs is pivotal for a normal spermatogenesis.

Caffeine is known to increase cells' metabolic rates, as well as the concentrations of free fatty acids and blood glucose [22, 23]. Animal studies suggest that prolonged exposures to caffeine may affect cells metabolism, compromising cellular homeostasis [24]. Within the testis, SCs produce lactate at high rates and any deregulation of this process may lead to high levels of oxidative stress (OS) and consequently male subfertility or infertility [25]. Interestingly, caffeine has been reported to be a protective substance against cellular damage with beneficial antioxidant effects [26]. The exact mechanisms of action of caffeine in SCs metabolism are yet to be disclosed and there is no evidence of a clear association between caffeine, OS and male fertility. Herein we hypothesize that caffeine can alter human SCs (hSCs) glycolytic and oxidative profile interfering with male reproductive potential.

Material and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specifically stated.

Patient selection and ethical issues

The patients' clinical study and testicular tissue processing was performed at the Centre for Reproductive Genetics Professor Alberto Barros (Porto, Portugal) according to local, national,

and European ethical committees' guidelines and the Declaration of Helsinki. Testicular biopsies were obtained from patients under treatment for recovery of male gametes and used after informed written consent. Only cells left in the tissue culture plates after patient's treatment were used. hSCs were isolated from six testicular biopsies of men with conserved spermatogenesis, selected from patients with anejaculation (psychological, vascular, neurologic), vasectomy or traumatic section of the vas deferens.

Human Sertoli cell primary culture

Testicular biopsies were washed twice in Hanks balanced salt solution (HBSS) through centrifugations at 500x*g* at room temperature (RT), as described by Oliveira and collaborators [27]. hSCs were obtained by a routine method [28]. The resulting cellular pellet was suspended in hSCs culture medium (Dulbecco's modified eagle medium:Ham's F12 nutrient mixture (DMEM:F12) 1:1, containing 15 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), 50 U/ml penicillin and 50 mg/ml streptomycin sulfate, 0.5 mg/ml fungizone, 50 µg/ml gentamicin and 10% heat inactivated fetal bovine serum (FBS)), and forced through a 20G needle to disaggregate large cell clusters. Then, cells were plated on Cell+ culture flasks (Sarstedt, Nümbrecht, Germany) and incubated at 30–33°C, 5% CO_2 until use. After 96 h, the cultures were examined by phase contrast microscopy and only the hSCs with contaminants below 5% were used. hSCs culture purity was determined as previously described [29].

Experimental groups

hSCs could grow until reach 90-95% of confluence, and after fully washed, the culture medium was replaced by serum-free medium (DMEM:F12 1:1, pH 7.4) supplemented with insulin–transferrin–sodium selenite (ITS; 10 mg/ml – 5 mg/ml – 5 µg/ml, respectively). To evaluate the effect of caffeine on hSCs glycolytic and oxidative profile, four different groups were defined: a control group without caffeine and three other groups containing ITS medium supplemented with increasing doses of caffeine (5, 50 and 500 µM). The daily concentration of caffeine between drinkers of caffeine-rich beverages was estimated to be 5 mg/kg of body weight [3, 6]. The intake of such amount of caffeine leads to a plasma concentration of about 10 µg/mL (50 µM) [7, 8], the reason why we choose to study the effects of 50 µM of caffeine in hSCs metabolism. Moreover, we found pertinent to evaluate the effects of a lower concentration (5 µM) and a higher concentration (500 µM), as caffeine content (and hence consumption) differs among the various caffeine-containing beverages and foodstuffs. After the 24 h of treatment, culture medium was collected. Then, cells were detached from the flask using a trypsin-ethylenediamine tetraacetic acid (EDTA) solution, counted with a Neubauer chamber and collected for protein extraction. Viability evaluated by the trypan blue exclusion test averaged 85–90%.

Proton nuclear magnetic resonance spectroscopy

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were acquired as previously described [30]. Sodium fumarate (final concentration of 10 mM) was used as internal reference (6.50 ppm) to quantify the following metabolites present in solution (multiplet, ppm): lactate (doublet, 1.33); alanine (doublet, 1.45) and H1-α-glucose (doublet, 5.22). The relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSproTM NMR spectral analysis program (Acorn, NMR Inc., Fremont, CA, USA).

Western blot

Total proteins were isolated from hSCs using radioimmunoprecipitation assay (RIPAS) buffer (1x phosphate-buffered saline (PBS), 1% NP-40, 0.5% sodium deoxycholate, 0.1% Sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride (PMSF), supplemented with 1% protease inhibitor cocktail, aprotinin and 100 mM sodium orthovanadate). Western blot was performed as previously described [31]. The resulting membranes were incubated overnight at 4°C with goat anti-glucose transporter 3 (GLUT3) (1:200, sc-7582, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-glucose transporter 1 (GLUT1) (1:200, sc-7903, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-phosphofructokinase 1 (PFK1) (1:500, sc-67028, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-monocarboxylate transporter 4 (MCT4) (1:1000, sc-50329, Santa Cruz Biotechnology, Heidelberg, Germany), or rabbit anti-lactate dehydrogenase (LDH) (1:10000, ab52488, Abcam, Cambridge, UK) primary antibodies. Mouse anti-α-tubulin (1:5000, T6199, Sigma Aldrich, St. Louis, MO, USA) was used as the protein loading control. The immunoreactive proteins were detected separately and visualized with goat anti-rabbit IgG-alkaline phosphatase (AP) (1:5000, sc-2007, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-goat IgG-AP (1:5000, A4187, Sigma-Aldrich, St. Louis, MO, USA) or goat anti-mouse IgG-AP (1:5000, sc-2008, Santa Cruz Biotechnology). Membranes were reacted with ECF™ (GE, Healthcare, Buckinghamshire, UK) and read with the BioRad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK). Densities from each band were obtained with BIO-PROFIL Bio-1D Software from Quantity One (Vilber Lourmat, Marne-la-Vallée, France) according to standard methods [32]. The band density attained was divided by the corresponding α-tubulin band intensities and expressed in fold variation relative to the control group.

Lactate dehydrogenase enzymatic assay

LDH levels were spectrophotometrically determined using a LDH enzymatic assay kit (Thermo Scientific, Waltham, MA) according to the manufacturer instructions. In brief, 5 µg of proteins were diluted in lysis buffer. Likewise, a blank was prepared and boiled for 5 min at 90°C for protein denaturation. LDH assay substrate was added to all samples in a dark environment and left at RT for approximately 15 min. Then, a stop solution was used to end the enzymatic activity and absorbance at 490 nm was measured using an Anthos 2010 microplate reader (Biochrom, Berlin, Germany). LDH enzymatic activities were calculated as units per milligram

of protein using the molar extinction factor (ε) and final expressed as fold variation to the control group.

Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) of the cellular pellets was determined using the colorimetric method described by Benzie and Strain [33]. In brief, working FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 2,4,6-Tripyridyl-s-Triazine (TPTZ) (10 mM in 40 mM HCl) and $FeCl_3$ (20 mM) in a 10:1:1 ratio (v:v:v). The reduction of the Fe^{3+}-TPTZ complex to a colored Fe^{2+}-TPTZ complex by the samples was monitored immediately after adding the sample and 40 min later, by measuring the absorbance at 595 nm using an Anthos 2010 microplate reader (Biochrom, Berlin, Germany). The antioxidant potential of the samples was determined against standards of ascorbic acid, which were processed in the same manner as the samples. Absorbance results were corrected by using a blank, with water instead of sample. The changes in absorbance values of test reaction mixtures were used to calculate FRAP value as described elsewhere [33].

Analysis of carbonyl groups and lipid peroxidation

Protein carbonyl content is commonly used as a marker for protein oxidation, while lipid peroxidation can be evaluated by measuring some resulting aldehydic products such as 4-hydroxynonenal (4-HNE). The content of protein carbonyl groups and 4-HNE in hSCs from the different experimental groups was evaluated using the slot-blot technique and specific antibodies. For carbonyl groups evaluation, protein samples were derivatized using 2,4-dinitrophenylhydrazine (DNPH) to obtain 2,4-dinitrophenol (DNP) according to the method developed by Levine and collaborators [34]. The slot-blot technique was performed using a Hybrid-slot manifold system (Biometra, Göttingen, Germany) and the resulting polyvinylidene fluoride (PVDF) membranes were incubated overnight (4°C) with a rabbit anti-DNP (1:5000, D9656, Sigma-Aldrich, St. Louis, MO, USA). For lipid peroxidation analysis, protein samples were diluted to a concentration of 0.001 µg/µL using PBS and transferred to activated PVDF membranes in the slot-blot technique. Then, the resulting membranes were incubated overnight (4°C) with a goat anti-4-HNE antibody (1:5000, AB5605, Merck Millipore, Temecula, USA). Membranes were then incubated with rabbit anti-goat IgG-AP (1:5000, A4187, Sigma-Aldrich, St. Louis, MO, USA) or goat anti-rabbit IgG-AP (1:5000, sc-2007, Santa Cruz Biotechnology, Heidelberg, Germany) secondary antibodies, respectively. Membranes were reacted with ECF™ substrate (GE Healthcare, Buckinghamshire, UK) and read using a BioRad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK). Densities from each band were quantified using the BIO-PROFIL Bio-1D Software from Quantity One (Vilber Lourmat, Marne-la-Vallée, France).

Statistical analysis

Statistical significance was assessed by one-way ANOVA, followed by Dunn post-test using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). All data were presented as mean ± SEM. Differences with $p < 0.05$ were considered statistically significant.

Results

Caffeine (50 μM) increases glucose transporters protein expression in human Sertoli cells

As caffeine is known to increase cells metabolic rates [22, 23], we hypothesized that it could also alter hSCs metabolism, thus affecting male fertility. For that purpose, we choose key points of hSCs metabolism starting on their primary energy substrate, glucose. Our results showed a glucose consumption of 10.7 ± 2.4 pmol/cell in hSCs from the control group (Figure 3.4A). Similarly, hSCs of the groups exposed to 5, 50 and 500 μM of caffeine consumed 6.9 ± 1.4, 7.0 ± 2.6 and 12.4 ± 3.1 pmol/cell, respectively (Figure 3.4A). In these cells, transport of glucose through the cytoplasmic membrane is mediated by specific membrane hexose transporters (mainly GLUT1 and GLUT3). Our results showed that the exposure of hSCs to 5 μM of caffeine did not alter the protein expression levels of GLUT1 or GLUT3 when compared to the control group. However, hSCs exposed to 50 μM of caffeine showed increased GLUT1 (1.30 ± 0.16-fold variation to control) (Figure 3.4B) and GLUT3 (1.31 ± 0.31-fold variation to control) protein levels (Figure 3.4C) relative to the control group. Moreover, in cells exposed to 50 μM of caffeine, GLUT3 protein expression was also higher relative to cells exposed to 5 μM of caffeine (Figure 3.4C). In hSCs exposed to 500 μM of caffeine, there were no differences in the protein expression levels of GLUT1 (Figure 3.4B) and GLUT3 (Figure 3.4C) (1.15 ± 0.09 and 1.10 ± 0.25-fold variation to control, respectively) relative to control or to the other concentrations of caffeine. The representative blots of GLUT1 and GLUT3 are shown in Figure 3.4D.

Lower caffeine concentrations reduced PFK1 protein levels while the highest caffeine concentration stimulated LDH activity

After glucose enters the cells, a key regulatory step of glycolytic pathway is mediated by PFK1 that irreversibly converts fructose 6-phosphate to fructose 1,6-bisphosphate. This latter is responsible for the activation of the enzyme pyruvate kinase that catalyzes the irreversible conversion to pyruvate. Our results showed that the lowest concentrations of caffeine (5 and 50 μM) decreased PFK1 protein levels to 0.65 ± 0.02 and 0.74 ± 0.04-fold variation to control, respectively (Figure 3.5A). Interestingly, exposure to the highest caffeine concentration led to PFK1 protein levels similar to the control group. The formed pyruvate can then be converted to lactate by LDH action. Our results demonstrated that LDH protein levels were similar between groups of hSCs treated with 5 μM (0.94 ± 0.19-fold variation to control), 50 μM (0.90 ± 0.15-fold variation to control) and 500 μM (0.91 ± 0.18-fold variation to control) of caffeine, showing no alterations comparative to the control group (Figure 3.5B).

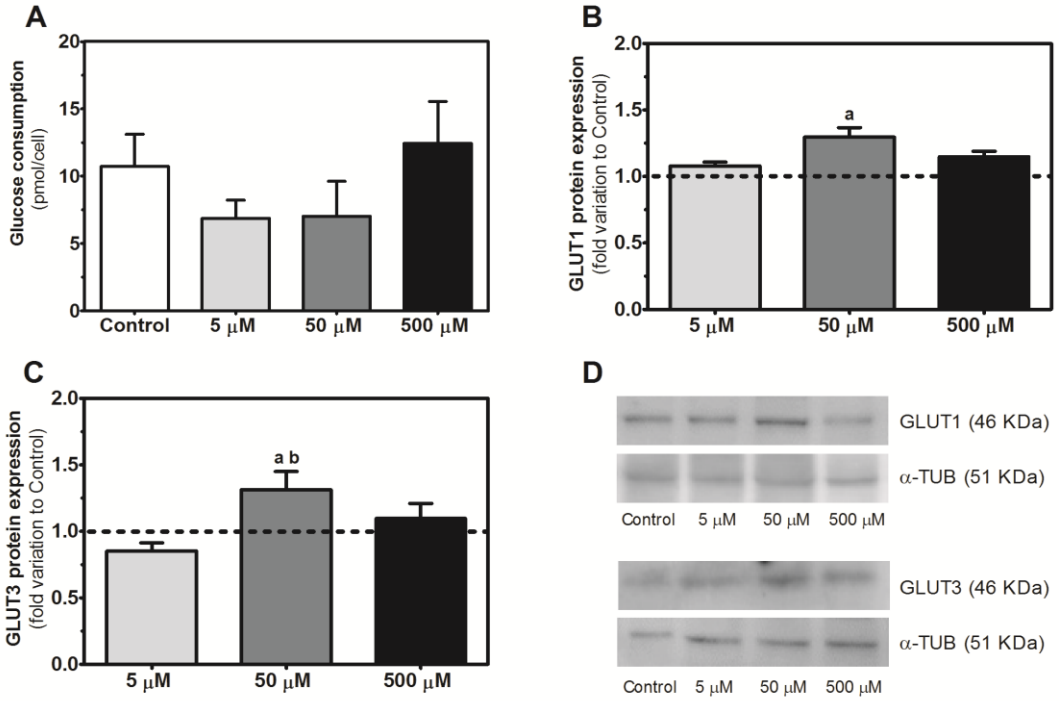


Figure 3.4 Effect of caffeine (5, 50 and 500 μM) on glucose consumption (Panel A) and glucose transporters (GLUT1 and GLUT3) protein expression (Panel B and C) in human Sertoli cells. The figure shows pooled data of independent experiments. Representative blots are also presented (Panel D). Glucose consumption is presented in pmol/cell. Variation in protein levels are presented as fold variation to control. Results are expressed as mean ± SEM (n = 5 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to control; b – relative to 5 μM.

As LDH protein expression levels in hSCs were not altered by caffeine action, we hypothesized that caffeine could alter the activity of this enzyme. In fact, we verified a dose-dependent increase in LDH activity of hSCs cultured with caffeine. The levels of LDH enzymatic activity in groups of hSCs exposed to 5, 50 and 500 μM of caffeine were 1.26 ± 0.16, 1.30 ± 0.16 and 1.49 ± 0.08-fold variation to control, respectively (Figure 3.5C). Nevertheless, only the highest concentration of caffeine (500 μM) increased LDH activity relative to the control group. After lactate production by SCs, it is exported through MCT4 to be used as metabolic fuel by developing germ cells. The MCT4 protein levels in hSCs exposed to 5, 50 and 500 μM of caffeine were 1.08 ± 0.12, 0.95 ± 0.10 and 0.93 ± 0.09-fold variation to control, respectively (Figure 3.5D). These values did not reach statistical significance when compared to the values determined in non-exposed cells. Figure 3.5E displays the representative blots of PFK1, LDH and MCT4.

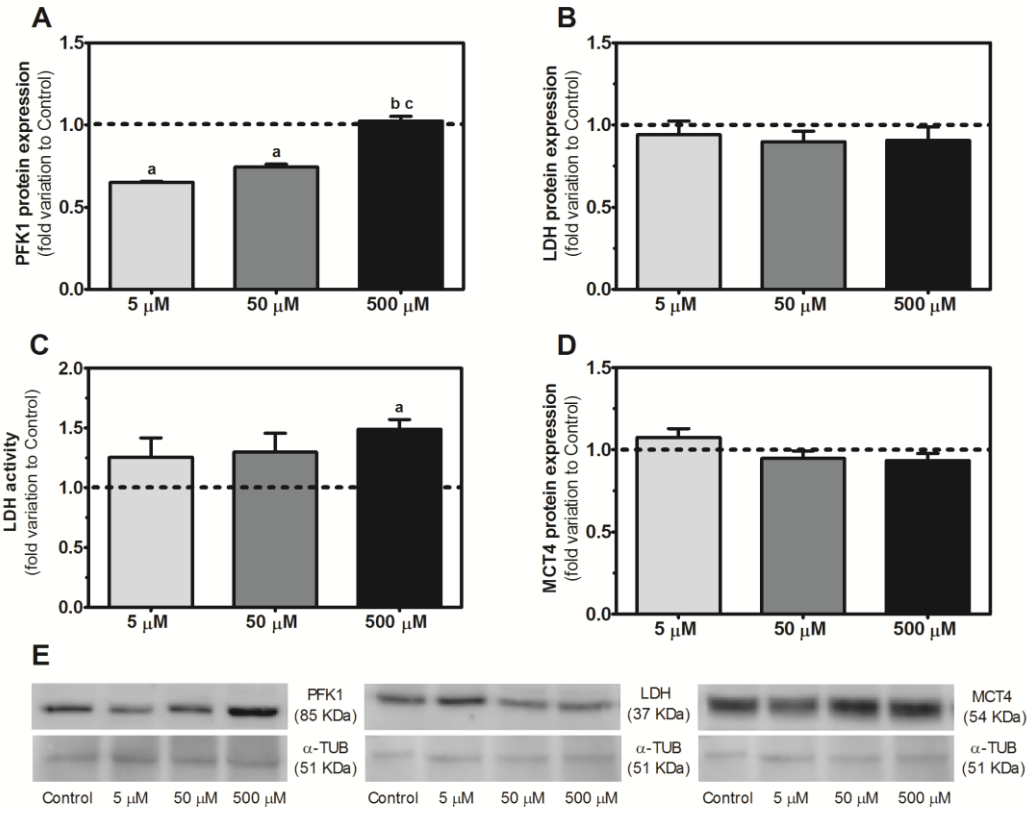


Figure 3.5 Effect of caffeine (5, 50 and 500 μM) in the expression of phosphofructokinase 1 (PFK1) (Panel A), lactate dehydrogenase (LDH) (Panel B) and monocarboxylate transporter 4 (MCT4) (Panel D), as well as LDH activity (Panel C) in human Sertoli cells. The figure shows pooled data of independent experiments. Representative blots are also presented (Panel E). Results are presented as fold variation to control and as mean ± SEM (n = 5 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to control; b – relative to 5 μM; c – relative to 50 μM.

Lactate production is stimulated by the lowest caffeine concentrations while alanine production is only stimulated by exposure to 50 μM of caffeine

Lactate production is one of the key functions of hSCs. Non-exposed hSCs presented a lactate production of 17.6 ± 2.4 pmol/cell. Interestingly, exposure to 5 and 50 μM of caffeine increased lactate production to 25.7 ± 2.2 pmol/cell and 26.3 ± 1.1 pmol/cell, respectively (Figure 3.6A). However, hSCs exposed to the highest concentration of caffeine (500 μM) did not show alterations in lactate production when compared with non-exposed cells. Alanine metabolism is closely associated with lactate production, as pyruvate can also be converted to alanine. Our results showed that extracellular alanine concentration in non-exposed cells was 0.51 ± 0.08 pmol/cell (Figure 3.6B). Exposure to 5 μM of caffeine did not alter alanine production (0.82 ± 0.08 pmol/cell) relative to the control group. On the other hand, there was an increase in alanine production in hSCs exposed to 50 μM of caffeine (0.88 ± 0.05 pmol/cell) relative to the control group (Figure 3.6B). hSCs exposed to the highest concentration of caffeine (500 μM) produced 0.92 ± 0.22 pmol/cell of alanine, having no differences in comparison to the other experimental groups. We also evaluated the ratio lactate/alanine, which is often used as an

index of the redox state of the cell [35]. The interconversion lactate – pyruvate - alanine is nicotinamide adenine dinucleotide reduced (NADH)-dependent and the ratio between the NADH and its oxidized form (NAD^+) can be estimated by the ratio lactate/alanine, which is often used as a measure of the cellular redox state [36]. Interestingly, our results showed that hSCs exposure to any of the caffeine concentrations studied did not alter the lactate/alanine ratio (Figure 3.6C).

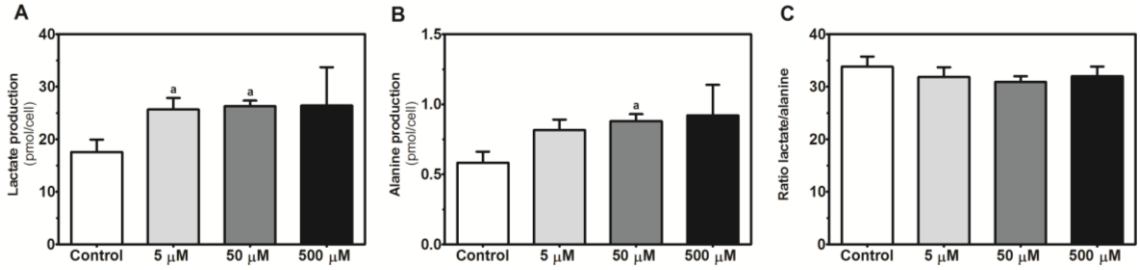


Figure 3.6 Effect of caffeine (5, 50 and 500 μM) in the production of lactate (Panel A) and alanine (Panel B), as well as the lactate/alanine ratio (Panel C) in human Sertoli cells. Metabolites production is presented as pmol/cell. The figure shows pooled data of independent experiments. Results are presented as mean ± SEM (n = 5 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to control.

Caffeine (50 μM) decreased protein oxidation and lipid peroxidation in human Sertoli cells, while the highest concentration induced a pro-oxidant environment

The high glycolytic rates observed in hSCs can lead to increased levels of OS. Several studies have reported that caffeine consumption is associated with reduced levels of OS biomarkers [26]. This was attributed to the antioxidant activity of caffeine [37]. We measured the antioxidant potential of hSCs pellets using the FRAP assay. The reducing power of a compound/extract serves as an indicator of its potential antioxidant activity (FRAP value). hSCs exposed to 5 μM of caffeine maintained a similar FRAP value (0.9 ± 0.2 μmol of antioxidant potential/μg of protein) as the control group (0.8 ± 0.2 μmol of antioxidant potential/μg of protein). However, hSCs exposed to 50 μM showed a decrease in the FRAP value to 0.6 ± 0.1 μmol of antioxidant potential/μg of protein, when compared to non-exposed cells and those exposed to 5 μM of caffeine. Moreover, hSCs exposed to 500 μM of caffeine presented a negative antioxidant potential value (-0.4 ± 0.2 μmol of antioxidant potential/μg of protein), indicating a pro-oxidant effect (Figure 3.7A).

Protein carbonylation and lipid peroxidation are strong biomarkers of OS. The attack of free radicals to proteins and membrane unsaturated fatty acids originates several products, such as DNP and 4-HNE, respectively, which can be measured to obtain a quantification of the cellular oxidative damages. hSCs exposed to 5 μM of caffeine presented a higher production of protein carbonyl groups (1.38 ± 0.11-fold variation) comparatively to the control group. In hSCs exposed to 50 μM of caffeine there was a decrease on the production of carbonyl groups (0.85 ± 0.12-fold variation to control) in comparison to hSCs exposed to 5 μM of caffeine. Finally, hSCs

exposed to 500 μM of caffeine presented a content in carbonyl groups of 1.47 ± 0.07-fold variation to control, which was higher relative to non-exposed cells and cells exposed to 50 μM of caffeine (Figure 3.7B). Concerning lipid peroxidation, there were no alterations on hSCs exposed to caffeine when compared with non-exposed cells. The lipid peroxidation values determined in hSCs exposed to 5, 50 and 500 μM were 1.12 ± 0.07, 0.87 ± 0.02 and 1.00 ± 0.06-fold variation to control, respectively. Nonetheless, there was a decrease in lipid peroxidation resultant from exposure of hSCs to 5 μM of caffeine relative to cells exposed to 50 μM (Figure 3.7C).

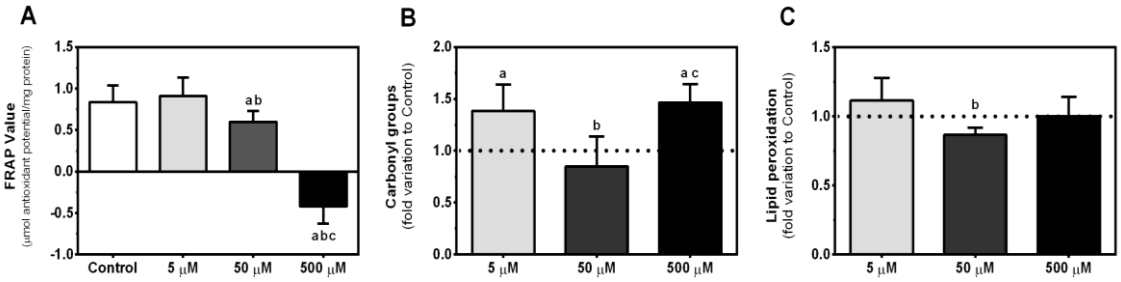


Figure 3.7 Effect of caffeine (5, 50 and 500 μM) in the ferric reducing antioxidant power (FRAP) value (Panel A), carbonyl groups formation (Panel B), and lipid peroxidation (Panel C) in human Sertoli cells. FRAP value is presented in μmol antioxidant potential/mg of protein, while carbonyl groups and lipid peroxidation are presented as fold variation to control. The figure shows pooled data of independent experiments. Results are presented as mean ± SEM (n = 5 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to control; b – relative to 5 μM; c – relative to 50 μM.

Discussion

Due to its wide consumption, caffeine potential health effects have been a focus of several studies. Popular beverages such as coffee, tea and energy drinks are known to have high concentrations of caffeine [10]. The average daily consumption of caffeine per individual was estimated to be of 5 mg/kg, reaching a concentration of 50 μM in the plasma [7, 38]. Therefore, we tested the effect of that concentration in hSCs glycolytic and oxidative profile to mimic the concentrations commonly found in plasma in *in vivo* conditions. However, people are commonly exposed to caffeine in lower and higher concentrations, therefore we also tested the effects of 5 and 500 μM of caffeine. Previous studies reported that caffeine increases human metabolic rates [39] and blood glucose levels [40]. Spermatogenesis is highly dependent on SCs glucose metabolism, as it results in the production of lactate, which is the preferred metabolic substrate of the developing germ cells (for review [41]). Thus, we evaluated the effect of caffeine in glucose metabolism of hSCs.

Caffeine is structurally very similar to adenosine and can bind to adenosine receptors acting as a non-selective antagonist [42]. Adenosine receptors have been identified in SCs [43]. Several follicle-stimulating hormone (FSH)-stimulated actions on SCs, such as inhibin and transferrin secretion and pyruvate metabolism, are mediated by A1 receptors [18, 44]. Moreover, adenosine promotes lactate supply to germ cells [45]. Our results showed that caffeine induces

a dose-dependent alteration in hSCs metabolism. Notably, exposure of hSCs to 5 μM and 50 μM increased lactate production, without changing glucose consumption. These results are concomitant with previous studies showing that caffeine can regulate glucose metabolism [46]. Glucose uptake by hSCs exhibits a strict hormonal regulation [47]. Moreover, these mechanisms are modulated by a direct regulatory effect in total GLUTs levels [48]. In fact, it has been described that hSCs can adapt their metabolism under certain conditions. For instance, in insulin or glucose deprivation conditions, hSCs adapt their metabolism by modulating the expression of GLUT1 and GLUT3 [35, 49]. In our experiments, when hSCs were exposed to 50 μM of caffeine there was an increase in the expression levels of these transporters, indicating a stimulation of glucose uptake. As these cells present an elevated glycolytic flux under normal conditions (for review [41]), glucose consumption was not altered. Nevertheless, exposure to the lowest concentrations of caffeine (5 and 50 μM) increased the glycolytic flux of hSCs, as demonstrated by the increase in lactate production by these cells. As adenosine is known to promote lactate supply to germ cells [45], our results showed that caffeine at these concentrations may mediate its effect in SCs metabolism by acting as antagonist of adenosine receptors.

When hSCs were exposed to 500 μM of caffeine, which is a high dose even for heavy drinkers of caffeine-rich beverages, we only detected alterations in PFK1 levels and LDH activity. Nevertheless, lactate production was not altered, nor glucose consumption. As referred, SCs exhibit a metabolic plasticity under stressful conditions (for review [21]). When subjected to glucose [49] or insulin [35] deprivation these cells adapt their metabolism in order to sustain a proper production of lactate, necessary for germ cell development. Thus, our results illustrate that caffeine at high doses (500 μM) alters SCs physiologic functions, however, these cells present some adaptive mechanisms, such as increased PFK1 expression and LDH activity, which allow them to maintain the same lactate production as normal cells.

Caffeine has been reported as a protective substance against cellular damage with beneficial antioxidant effects [26, 50]. Compelling evidence has shown that caffeine and adenosine receptors can modulate cellular oxidant status [51, 52]. Our results show that at high concentrations (500 μM), caffeine induces a pro-oxidant environment in hSCs, thus explaining the requirement for the reported metabolic adaptations in hSCs to sustain lactate production. Moreover, hSCs exposed to 500 μM of caffeine presented a higher protein oxidation, as illustrated by the increase in carbonyl groups content relative to control group and cells exposed to 50 μM. The effect of caffeine in several cellular functions, such as reactive oxygen species (ROS) generation, was reported as concentration-specific [53]. Although caffeine is generally known for its antioxidant properties and efficiency as an hydroxyl radical scavenger *in vitro* [54], our results showed that at a concentration of 500 μM, caffeine induces a pro-oxidant environment in SCs that is accompanied by an increase in proteins oxidation. The lowest dose of caffeine tested (5 μM) did not alter the antioxidant capacity of hSCs when compared to non-exposed cells. However, there was an increase in protein oxidation, evidencing an increase

in OS. hSCs exposed to 50 μM of caffeine presented lower antioxidant capacity than non-exposed cells and cells exposed 5 μM of caffeine. However, hSCs from this group (50 μM of caffeine) presented lower lipid peroxidation than non-exposed cells and lower protein oxidation than hSCs exposed to 5 μM of caffeine. These results support that the effects of caffeine in OS depend on the concentration. The intermediate concentration (50 μM), which corresponds to the regular caffeine ingestion, appears to mediate some protective effects against OS. As discussed, caffeine can act as a nonselective antagonist of adenosine receptors, which are reported to control the formation of free radicals in cells [52]. Thus, the effects of caffeine in OS may also be mediated by adenosine receptors, which would be concomitant with the changes detected in hSCs metabolism.

Concerning the effects of caffeine in the male reproductive function, there are some conflicting results. Maternal caffeine consumption has been reported to impair gonadal development and induce several long-term adverse effects on the reproductive health of the offspring [55]. More specifically, it leads to decreases in testicular weight, seminiferous tubules diameter, germinal epithelium height, testosterone levels and sperm quality of the offspring [55]. Besides, an in vivo study with Sprague-Dawley rats demonstrated that and oral administration of an elevated dose of 200 mg/kg of body weight of caffeine negatively affects the histo-architecture of the seminiferous tubules of the testis, with massive loss of spermatogenic cells and testicular weight loss [56]. A more recent study with the same dosage of caffeine (200 mg/kg of body weight) showed testicular and epididymal weight loss, as well as histological alterations, i.e., atrophic cells with necrosis and excessive degeneration of spermatids and almost absence of spermatozoa [57]. Of note, caffeine has also been used to induce sperm capacitation, particularly with frozen thawed spermatozoa [58]. When human spermatozoa were incubated with caffeine, it was reported that the rate of glycolysis increased, without changing adenosine diphosphate (ADP) and adenosine triphosphate (ATP) levels [59]. Testicular metabolism is crucial for spermatogenesis [20, 60]. The strict metabolic cooperation established between SCs and developing germ cells is sensitive to hormonal fluctuations [36] and other factors [61]. Recently, we have reported that a white tea extract, which is very rich in caffeine [61], is a promising antioxidant medium additive for sperm storage at RT. Supplementation with a white tea extract increased sperm antioxidant potential and decreased lipid peroxidation, maintaining spermatozoa viability to values similar to those obtained at collection time [62]. We also showed that a white tea extract can modulate SCs and stimulate lactate production by these cells [63]. These potential benefits in male reproductive health were attributed to several phytochemicals, including the high concentration of caffeine. In fact, caffeine has been consistently reported as a metabolic modulator. Our results show that caffeine modulates hSCs metabolism in a dose-dependent manner. The lowest caffeine concentration stimulates lactate production without interfering with the glycolysis-related machinery studied. However, the concentration of 50 μM, altered glucose transporters and increased alanine production to maintain the lactate/alanine ratio. This ratio is very important because it reflects the

intracellular redox state. Lactate has been reported to protect germ cells *in vivo* by suppressing the loss of spermatocytes and spermatids in cryptorchid rats [64]. Thus, it appears that caffeine at "normal-range" can enhance lactate production by hSCs and promote germ cells survival. Interestingly, the most effective concentration in preventing protein oxidation and lipid peroxidation in hSCs was 50 µM, which is a frequent concentration found in the plasma of moderate consumers of caffeine-rich beverages.

In sum, our results showed that caffeine is a modulator of hSCs metabolism and stimulates lactate production in a dose-dependent way. When exposed to a high concentration of caffeine, hSCs present a pro-oxidant environment and crucial adaptations in their metabolism to sustain lactate production. Moreover, it promotes proteins oxidation in hSCs. These results illustrate that moderate consumption of caffeine appears to be safe or even positive to the metabolic functioning of hSCs. Nevertheless, caution should be taken by consumers of energetic beverages and supplemented food with caffeine to avoid deleterious effects to hSCs functioning and spermatogenesis arrest.

References

1. Barone J and Roberts H (1996) Caffeine consumption. Food and Chemical Toxicology 34(1):119-29.
2. Heatherley S, et al. (2006) Caffeine consumption among a sample of UK adults. Appetite 47(2):266.
3. FSA (2004). Food Standards Agency - Survey of Caffeine levels in hot beverages. Food Survey information sheet 53/04,
4. Eteng M, et al. (1997) Recent advances in caffeine and theobromine toxicities: a review. Plant Foods for Human Nutrition 51(3):231-43.
5. Mukhtar H, et al. (1992) Tea components: antimutagenic and anticarcinogenic effects. Preventive Medicine 21(3):351-60.
6. Mandel H (2002) Update on caffeine consumption, disposition and action. Food and Chemical Toxicology 40(9):1231-34.
7. Blanchard J and Sawers S (1983) The absolute bioavailability of caffeine in man. European Journal of Clinical Pharmacology 24(1):93-98.
8. Alvi SN and Hammami MM (2011) Validated HPLC method for determination of caffeine level in human plasma using synthetic plasma: application to bioavailability studies. Journal of chromatographic science 49(4):292-96.
9. Sepkowitz KA (2013) Energy drinks and caffeine-related adverse effects. JAMA 309(3):243-44.
10. Reissig CJ, et al. (2009) Caffeinated energy drinks—a growing problem. Drug and Alcohol Dependence 99(1):1-10.
11. Andrews KW, et al. (2007) The caffeine contents of dietary supplements commonly purchased in the US: analysis of 53 products with caffeine-containing ingredients. Analytical and Bioanalytical Chemistry 389(1):231-39.
12. Benowitz NL (1990) Clinical pharmacology of caffeine. Annual Review of Medicine 41(1):277-88.
13. Dunwiddie TV and Masino SA (2001) The role and regulation of adenosine in the central nervous system. Annual Review of Neuroscience 24(1):31-55.
14. Fisone G, et al. (2004) Caffeine as a psychomotor stimulant: mechanism of action. Cellular and Molecular Life Sciences 61(7-8):857-72.
15. Casali EA, et al. (2001) Ectonucleotidase activities in Sertoli cells from immature rats. Brazilian Journal of Medical and Biological Research 34(10):1247-56.
16. Stiles GL, et al. (1986) The Rat Testicular Ai Adenosine Receptor-Adenylate Cyclase System. Endocrinology 119(4):1845-51.
17. Rivkees SA (1994) Localization and characterization of adenosine receptor expression in rat testis. Endocrinology 135(6):2307-13.
18. Conti M, et al. (1989) Characterization and Function of Adenosine Receptors in the Testis. Annals of the New York Academy of Sciences 564(1):39-47.
19. Mackay S (2000) Gonadal development in mammals at the cellular and molecular levels. International Review of Cytology 200:47-99.

20. Rato L, et al. (2012) Metabolic regulation is important for spermatogenesis. Nature Reviews Urology 9(6):330-38.
21. Alves M, et al. (2013) Exposure to 2, 4-dichlorophenoxyacetic acid alters glucose metabolism in immature rat Sertoli cells. Reproductive Toxicology 38:81-88.
22. Lane JD (2011) Caffeine, glucose metabolism, and type 2 diabetes. Journal of Caffeine Research 1(1):23-28.
23. Sinha RA, et al. (2014) Caffeine stimulates hepatic lipid metabolism by the autophagy-lysosomal pathway in mice. Hepatology 59(4):1366-80.
24. Yokogoschi H, et al. (1983) Hypercholesterolemic effects of caffeine-containing beverages and xanthine-derivatives in rats. Nutrition reports international 28(4):805-14.
25. Aitken RJ, et al. (2010) Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. Human Reproduction 25(10):2415-26.
26. Grucka-Mamczar E, et al. (2009) The influence of sodium fluoride and caffeine on the activity of antioxidative enzymes and the concentration of malondialdehyde in rat liver. Fluoride 42(2):105-09.
27. Oliveira PF, et al. (2011) Influence of 5α-dihydrotestosterone and 17β-estradiol on human Sertoli cells metabolism. International Journal of Andrology 34(6pt2):e612-e20.
28. Oliveira PF, et al. (2009) Intracellular pH regulation in human Sertoli cells: role of membrane transporters. Reproduction 137(2):353-59.
29. Steger K, et al. (1996) Immunohistochemical detection of immature Sertoli cell markers in testicular tissue of infertile adult men: a preliminary study. International Journal of Andrology 19(2):122-28.
30. Alves M, et al. (2011) Substrate selection in hearts subjected to ischemia/reperfusion: role of cardioplegic solutions and gender. NMR in Biomedicine 24(9):1029-37.
31. Dias TR, et al. (2013) Insulin deprivation decreases caspase-dependent apoptotic signaling in cultured rat sertoli cells. ISRN urology 2013.
32. Picado C, et al. (1999) Cyclooxygenase-2 mRNA is downexpressed in nasal polyps from aspirin-sensitive asthmatics. American Journal of Respiratory and Critical Care Medicine 160(1):291-96.
33. Benzie IF and Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical Biochemistry 239(1):70-76.
34. Levine RL, et al. (1990) Determination of carbonyl content in oxidatively modified proteins. Methods in Enzymology 186:464-78.
35. Oliveira PF, et al. (2012) Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells. Biochimica et Biophysica Acta 1820(2):84-89.
36. Alves MG, et al. (2013) Molecular mechanisms beyond glucose transport in diabetes-related male infertility. Biochimica et Biophysica Acta 1832(5):626-35.
37. Devasagayam T, et al. (1996) Caffeine as an antioxidant: inhibition of lipid peroxidation induced by reactive oxygen species. Biochimica et Biophysica Acta 1282(1):63-70.
38. Chou TM and Benowitz NL (1994) Caffeine and coffee: effects on health and cardiovascular disease. Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology and Endocrinology 109(2):173-89.
39. Nehlig A, et al. (1992) Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. Brain Research Reviews 17(2):139-70.
40. Pizziol A, et al. (1998) Effects of caffeine on glucose tolerance: a placebo-controlled study. European Journal of Clinical Nutrition 52(11):846-49.
41. Rato L, et al. (2014) Pre-diabetes alters testicular PGC1-α/SIRT3 axis modulating mitochondrial bioenergetics and oxidative stress. Biochimica et Biophysica Acta (BBA)-Bioenergetics 1837(3):335-44.
42. Fredholm BB and Lindström K (1999) Autoradiographic comparison of the potency of several structurally unrelated adenosine receptor antagonists at adenosine A_1 and A_{2A} receptors. European journal of pharmacology 380(2):197-202.
43. Monaco L and Conti M (1986) Localization of adenosine receptors in rat testicular cells. Biology of Reproduction 35(2):258-66.
44. Meroni S, et al. (1998) Effects of purinergic agonists on aromatase and gamma-glutamyl transpeptidase activities and on transferrin secretion in cultured Sertoli cells. The Journal of Endocrinology 157(2):275-83.
45. Galardo M, et al. (2010) Adenosine regulates Sertoli cell function by activating AMPK. Molecular and Cellular Endocrinology 330(1):49-58.
46. Ojuka EO, et al. (2002) Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca^{2+}. American Journal of Physiology, Endocrinology and Metabolism 282(5):E1008-E13.
47. Alves M, et al. (2013) Hormonal control of Sertoli cell metabolism regulates spermatogenesis. Cellular and Molecular Life Sciences 70(5):777-93.
48. Klip A, et al. (1994) Regulation of expression of glucose transporters by glucose: a review of studies in vivo and in cell cultures. The FASEB journal 8(1):43-53.
49. Riera MF, et al. (2009) Molecular mechanisms involved in Sertoli cell adaptation to glucose deprivation. American Journal of Physiology, Endocrinology and Metabolism 297(4):E907-E14.

50. Ofluoglu E, et al. (2009) The effects of caffeine on l-arginine metabolism in the brain of rats. Neurochemical Research 34(3):395-99.
51. Prasanthi JR, et al. (2010) Caffeine protects against oxidative stress and Alzheimer's disease-like pathology in rabbit hippocampus induced by cholesterol-enriched diet. Free Radical Biology and Medicine 49(7):1212-20.
52. Gołembiowska K and Dziubina A (2012) The Effect of Adenosine A_{2A} Receptor Antagonists on Hydroxyl Radical, Dopamine, and Glutamate in the Striatum of Rats with Altered Function of VMAT2. Neurotoxicity Research 22(2):150-57.
53. Tiwari KK, et al. (2014) Differential concentration-specific effects of caffeine on cell viability, oxidative stress, and cell cycle in pulmonary oxygen toxicity *in vitro*. Biochemical and biophysical research communications 450(4):1345–50.
54. Shi X, et al. (1991) Antioxidant behaviour of caffeine: efficient scavenging of hydroxyl radicals. Food and Chemical Toxicology 29(1):1-6.
55. Dorostghoal M, et al. (2012) Maternal caffeine consumption has irreversible effects on reproductive parameters and fertility in male offspring rats. Clinical and Experimental Reproductive Medicine 39(4):144-52.
56. Bassey RB, et al. (2011) Effects of Tahitian Noni dietary supplement on caffeine-induced testicular histo-pathological alterations in adult Sprague-Dawley rats. Middle East Fertility Society Journal 16(1):61-66.
57. Ekaluo U, et al. (2014) Protective role of soursop (Annona muricata L.) fruit on testicular toxicity induced by caffeine in albino rats. Journal of Life Sciences Research and Discovery 1:26-30.
58. Funahashi H and Nagai T (2001) Regulation of in vitro penetration of frozen–thawed boar spermatozoa by caffeine and adenosine. Molecular Reproduction and Development 58(4):424-31.
59. Rees JM, et al. (1990) Effect of caffeine and of pentoxifylline on the motility and metabolism of human spermatozoa. Journal of Reproduction and Fertility 90(1):147-56.
60. Dias TR, et al. (2014) Sperm glucose transport and metabolism in diabetic individuals. Molecular and Cellular Endocrinology 396(1–2):37-45.
61. Dias TR, et al. (2013) Glucose Transport and Metabolism in Sertoli Cell: Relevance for Male Fertility. Current Chemical Biology 7(3):282-93.
62. Dias TR, et al. (2014) White Tea as a Promising Antioxidant Medium Additive for Sperm Storage at Room Temperature: A Comparative Study with Green Tea. Journal of Agricultural and Food Chemistry 62(3):608-17.
63. Martins AD, et al. (2013) Effect of white tea (Camellia sinensis (L.)) extract in the glycolytic profile of Sertoli cell. European Journal of Nutrition 53(6):1383-91.
64. Courtens J and Plöen L (1999) Improvement of spermatogenesis in adult cryptorchid rat testis by intratesticular infusion of lactate. Biology of Reproduction 61(1):154-61.

Implications of epigallocatechin gallate in cultured human Sertoli cells glycolytic and oxidative profile

Abstract

Sertoli cells are crucial for the success of spermatogenesis, which is the biological process that ensures male fertility. These cells present high metabolic rates, being often subjected to high levels of reactive oxygen species that, if uncontrolled, may compromise male fertility. Once the most abundant tea catechin, epigallocatechin gallate (EGCG), has demonstrated a potent preventive activity against oxidative stress, we have evaluated its effect at concentrations of 5 and 50 μM, on the metabolism, mitochondrial functionality and oxidative profile of human Sertoli cells (hSCs). The lowest concentration of EGCG (5 μM) did not alter hSCs function. The highest concentration of EGCG (50 μM) increased glucose and pyruvate consumption, while decreasing the conversion of pyruvate to alanine to sustain a regular lactate production. However, despite maintaining Krebs cycle functionality, EGCG (50 μM) decreased mitochondrial membrane potential of hSCs, which could compromise the normal rates of adenosine triphosphate (ATP) production. Interestingly, oxidative damages to proteins and lipids decreased in this experimental group, which may be valuable for the success of spermatogenesis.

Keywords: epigallocatechin gallate; glucose metabolism; human Sertoli cells; mitochondrial bioenergetics; oxidative profile; spermatogenesis.

Introduction

Tea is one of the most widely consumed beverages worldwide. Although it is commonly drunk for pleasure, tea is well-known for its health-promoting properties. The main constituents of tea leaves include proteins, polyphenols, methylxanthines, amino and organic acids [1]. Epigallocatechin gallate (EGCG) is the most abundant and biologically active tea polyphenol, representing 50-80% of the total tea catechins [2]. Most of the tea's medicinal properties are attributed to EGCG, which has demonstrated a potent preventive activity against oxidative stress (OS) [3]. Its ability to protect cells from reactive oxygen species (ROS)-induced damages has made EGCG a popular nutraceutical. Tea and its constituents are currently receiving considerable attention as they can be used as dietary supplements to prevent or even treat several diseases/dysfunctions. For instance, they have been described as potential modulators of spermatogenesis [4], which is the process of sperm production. Sertoli cells (SCs) are the testicular cells responsible for the maintenance of spermatogenesis. The resulting metabolites of the distinctive metabolism of these cells are essential for the survival of developing germ cells. Particularly, the lactate produced by SCs constitutes the preferred substrate of germ cells for energy production [5]. When the metabolic cooperation between SCs and germ cells is

disrupted, fertility problems may arise [6]. The increasing incidence of male subfertility/infertility highlighted the need for new therapies. Interestingly, the consumption of tea seems to be a promising approach [7, 8]. However, the underlying mechanisms remain unknown. We have previously demonstrated that a white tea extract was able to alter rat SCs metabolism, suggesting possible implications for male fertility [9]. Additionally, the exposure of human SCs (hSCs) to the most abundant methylxanthine of tea, caffeine, led to increased production of lactate and alanine, affecting cells oxidative profile [10]. Though, there is a lack of information concerning the effect of catechins on these testicular cells. Herein, we aimed to evaluate the effect of EGCG (5 and 50 μM) on hSCs metabolism, mitochondrial functionality and oxidative profile. We hypothesized that EGCG can act as a relevant modulator of the nutritional support of spermatogenesis, being useful to counteract male reproductive problems associated with hSCs dysfunction.

Material and Methods

Chemicals

Dulbecco's modified eagle medium (DMEM), Ham's F12 nutrient mixture (F12) and Fetal bovine serum (FBS): Biochrom (Leonorenstr, Berlin, Germany); Mammalian Protein Extraction Reagent (M-PER) and lactate dehydrogenase (LDH) Enzymatic Assay Kit: Thermo Scientific (Waltham, MA, USA); 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC1): Molecular Probes (Eugene, OR, USA); EGCG (CAS Number 989-51-5): Sigma-Aldrich (St. Louis, MO, USA); Extracellular O_2 Consumption assay: Abcam (Cambridge, UK); All other chemicals: Sigma-Aldrich (St. Louis, MO, USA) unless specifically stated.

Human Sertoli cell primary culture

Testicular tissue processing was performed at Centre for Reproductive Genetics Professor Alberto Barros (Porto, Portugal) according to local, national, and European ethical committees' guidelines and the Declaration of Helsinki. Six testicular biopsies were obtained from six different patients with conserved spermatogenesis, after informed written consent. hSCs were obtained from the cells left in tissue culture plates after each patient's treatment and isolated by our routine method [11]. Cells from each individual were plated separately in six Cell+ culture flasks (Sarstedt, Nümbrecht, Germany) and incubated at 33-34°C to mimic the temperature to which hSCs are subjected *in vivo* into the scrotum [12]. Moreover, a controlled atmosphere of 5% CO_2 was maintained in the incubator, as it is required to stabilize the pH of the growth media. After 96 h, cultures were examined by phase contrast microscopy and only hSCs with contaminants below 5% were used. hSCs culture purity was determined as previously described [13].

Experimental groups

hSCs could grow until reaching 90-95% of confluence. Subsequently, the culture media were replaced by serum-free media (DMEM:F12, 1:1, pH 7.4) supplemented with insulin–transferrin–sodium selenite (ITS; 10 µg/mL – 5.5 µg/mL – 0.005 µg/mL, respectively). Three experimental groups were defined: a control group without EGCG and two groups supplemented either with 5 or 50 µM of EGCG. Although the bioavailability of EGCG after drinking tea is very low (0.3-0.5 µM) [14], the ingestion of 400-1200 mg of EGCG from a tea extract (fasting conditions) may result in plasma levels of 2-7 µM [15]. Based on these studies, we have selected the lowest concentration of 5 µM of EGCG. Furthermore, as many studies reported the pharmacological relevance of EGCG at 50 µM [16, 17], we have also included this concentration in our study. After 24 h of treatment with 0, 5 or 50 µM of EGCG, hSCs and the respective culture media were collected. Cell viability was evaluated by the trypan blue exclusion test and averaged 85-90%.

Sulforhodamine B assay

A sulforhodamine B (SRB) colorimetric assay was performed as previously described [18], to evaluate hSCs proliferative responses to the culture media [10]. In brief, hSCs were plated in a 96-well culture plate (same number of cells per well), left to grow until reaching a confluence of 60-70% and treated with the test culture media containing 0, 5 or 50 µM of EGCG for 24 h. Then, cells were fixed overnight (at -20°C) with a mixture containing 1% acetic acid and 99% methanol, and subsequently stained with 0.05% (w/v) SRB dissolved in 1% acetic acid for 1 h. Unbound SRB was removed by washing with 1% acetic acid, whereas the bound SRB was solubilized with 10 mM Tris base (pH 10) in a shaker for 10 min. A blank was prepared with Tris base (pH 10) and the absorbance was read at 492 nm. Absorbance readings of SRB-stained cells gives a direct measure of cell numbers. To obtain concentration–response curves we defined the cell growth of the control group as 100% and calculated the cell growth of treated groups relative to control.

Proton nuclear magnetic resonance spectroscopy

Proton nuclear magnetic resonance (^{1}H-NMR) spectra of hSCs extracellular culture media were acquired and quantified as described by our team [19]. Sodium fumarate (final concentration of 1 mM) was used as an internal reference (6.50 ppm) to quantify the following metabolites present in hSCs extracellular media (multiplet, ppm): lactate (doublet, 1.33); alanine (doublet, 1.45), acetate (singlet, 1.90), pyruvate (singlet, 2.35), citrate (multiplet, 2.57-2.72) and H1-α-glucose (doublet, 5.22). Relative areas of ^{1}H-NMR resonances and metabolites concentrations were quantified as described [19].

Western blot

Total proteins from hSCs were isolated using M-PER according to manufacturer's instructions. Western blot was performed as previously described [20]. In brief, proteins were fractionated in 12% polyacrylamide gels, then the separated proteins were transferred to previously activated polyvinylidene difluoride (PVDF) membranes and blocked for 90 min with a 5% non-fat milk solution at room temperature (RT). Afterwards, the membranes were incubated overnight at 4°C with the primary antibodies listed in Table 3.3. Mouse anti-ß-actin was used as the protein loading control. The immunoreactive proteins were detected separately and visualized after incubation with the respective secondary antibodies (Table 3.3) for 90 min, at RT. The band density attained was divided by the corresponding ß-actin band intensities and expressed in fold variation (induction/reduction) relative to the control group.

Table 3.3 List of the primary and secondary antibodies used in this study.

Antibody	Source	KDa	Dilution	Vendor	Catalog #
GLUT1	Rabbit	55	1:200	Santa Cruz Biotechnology Heidelberg, Germany	sc-7903
GLUT2	Rabbit	60-62	1:5000	Santa Cruz Biotechnology Heidelberg, Germany	sc-9117
GLUT3	Goat	48-70	1:200	Santa Cruz Biotechnology Heidelberg, Germany	sc-7582
PFK1	Rabbit	85	1:500	Santa Cruz Biotechnology Heidelberg, Germany	sc-67028
MCT4	Rabbit	43	1:1000	Santa Cruz Biotechnology Heidelberg, Germany	sc-50329
LDH	Rabbit	37-38	1:10000	Abcam Cambridge, MA, USA	ab52488
ß-Actin	Mouse	42	1:5000	Thermo Scientific Waltham, MA, USA	MA5-15739
DNP	Rabbit	---	1:5000	Sigma-Aldrich St. Louis, MO, USA	D9656
Nitro-Tyrosine	Rabbit	---	1:5000	Cell Signaling Technology Leiden, Netherlands	9691
4-HNE	Goat	---	1:5000	Merck Millipore Temecula, USA	AB5605
Total OXPHOS Cocktail	Mouse	CI-20 CII-30 CIII-48 CIV-40 CV-55	1:1000	MitoSciences Eugene, Oregon, USA	ab110413
Mouse	Goat	—	1:5000	Sigma-Aldrich St. Louis, MO, USA	A3562
Rabbit	Goat	—	1:5000	Sigma-Aldrich St. Louis, MO, USA	A3687
Goat	Rabbit	—	1:5000	Sigma-Aldrich St. Louis, MO, USA	A4187

Abbreviations: GLUT1, glucose transporter 1; GLUT2, glucose transporter 2; GLUT3, glucose transporter 3; PFK1, phosphofructokinase 1; MCT4, monocarboxylate transporter 4; LDH, lactate dehydrogenase; DNP, 2,4-dinitrophenol; 4-HNE, 4-hydroxynonenal; CI, NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 8 (NDUFB8); CII, succinate dehydrogenase complex, subunit B, iron sulfur (SDHB); CIII, ubiquinol-cytochrome c reductase core protein II (UQCRC2); CIV, mitochondrially encoded cytochrome c oxidase I (MTCO1); CV, ATP synthase alpha-subunit (ATP5A).

Lactate dehydrogenase enzymatic assay

Intracellular LDH activity levels of hSCs were spectrophotometrically determined using the LDH enzymatic assay kit as described [10]. Absorbance at 490 nm was measured using an Anthos 2010 microplate reader (Biochrom, Berlin, Germany). LDH enzymatic activities were calculated as units per milligram of protein, using the molar extinction factor (ε) and expressed as fold variation to the control group.

Mitochondrial membrane potential

The fluorescent probe JC1 was used to measure mitochondrial membrane potential of hSCs, using a slightly modified version of the method described by Salvioli and collaborators [21]. The accumulation of the JC1 dye in mitochondria depends on mitochondrial membrane potential. In brief, hSCs were cultured in a 96-well culture plate (same number of cells per well) with DMEM:F12 (1:1, pH 7.4) supplemented with 1% FBS. Cells were left to grow until reach 60-70% of confluence. Then, the culture medium was replaced by ITS medium supplemented with 0, 5 or 50 µM of EGCG. After 24 h, the medium was removed, and cells were washed with phosphate-buffered saline (PBS). A total of 100 µL of JC1 staining solution (1µg/mL), previously prepared in DMEM:F12 supplemented with 1% FBS, was added to each well and cells were incubated for 15 min at 37°C. Afterwards, cells were washed with PBS and finally 100 µL of DMEM:F12 supplemented with 1% FBS were added to each well. Fluorescence intensities were analyzed immediately using a Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek, Winooski, USA) pre-heated at 37°C. Cells with functional mitochondria exhibited JC1 aggregates that were detected at 550/590nm (excitation/emission), while cells with dysfunctional mitochondria mainly exhibited JC1 monomers detected at 485/535nm (excitation/emission). The JC1 ratio aggregates/monomers was calculated for each condition as a measure of mitochondrial functionality.

Extracellular oxygen consumption assay

Oxygen consumption is one of the most informative and direct measures of mitochondrial function. We used the extracellular oxygen consumption assay kit (ab197243; Abcam, Cambridge, UK), according to the manufacturer's instructions, to measure the respiration of hSCs after exposure to EGCG. Briefly, hSCs were cultured in a 96-well culture plate with DMEM:F12 (1:1, pH 7.4) supplemented with 10% FBS. Cells were left to grow until reach 80-90% of confluence. Then, the medium was replaced by ITS medium supplemented with 0, 5 or 50 µM of EGCG. After 24 h of exposure, the medium was removed and replaced by freshly prepared ITS medium. Extracellular O_2 consumption reagent (10 µL) was added to each well, except to the blank control. Two drops of high-sensitivity mineral oil (pre-heated at 37°C) were added to each well to limit back diffusion of ambient oxygen. Fluorescence intensities were analyzed immediately using a Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek, Winooski, USA) pre-heated at 37°C. Extracellular oxygen consumption of hSCs was measured at 1.5 min intervals for 120 min at Excitation/Emission=380/650nm. Cells respiration leads to oxygen depletion from

the surrounding environment, resulting in the increase in fluorescence signal. Fluorescence intensities were normalized to the blank and expressed as counts per second (CPS) *versus* time (min).

Analysis of carbonyl groups, nitration and lipid peroxidation

Carbonyl groups, nitration and lipid peroxidation are usually used as biomarkers for oxidation and can be evaluated by measuring its resulting products, 2,4-dinitrophenol (DNP), nitro-tyrosine and 4-hydroxynonenal (4-HNE), respectively. The content of these adducts in hSCs after exposure to EGCG was evaluated using specific antibodies (Table 3.3) by slot-blot as previously described [8, 10]. Results were expressed as fold variation to the control group.

Statistical analysis

Statistical significance was assessed by one-way ANOVA, followed by Dunn post-test using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). All data are presented as mean ± SEM (n = 6). Differences with $p < 0.05$ were considered statistically significant.

Results

Exposure to the highest dose of EGCG (50 µM) decreased hSCs proliferation

We evaluated hSCs proliferation after exposure to the several doses of EGCG by the SRB assay. Our results demonstrated that addition of 5 µM of EGCG to hSCs culture medium did not alter hSCs growth (77 ± 15%) when compared to the control group (100 ± 16%) (Figure 3.8). However, exposure of hSCs to 50 µM of EGCG induced a decrease in cell proliferation (34 ± 11%) to almost one-third of that observed in the control group (Figure 3.8). Based on these results, all the subsequent results were normalized for the total number of cells or amount of protein in each sample.

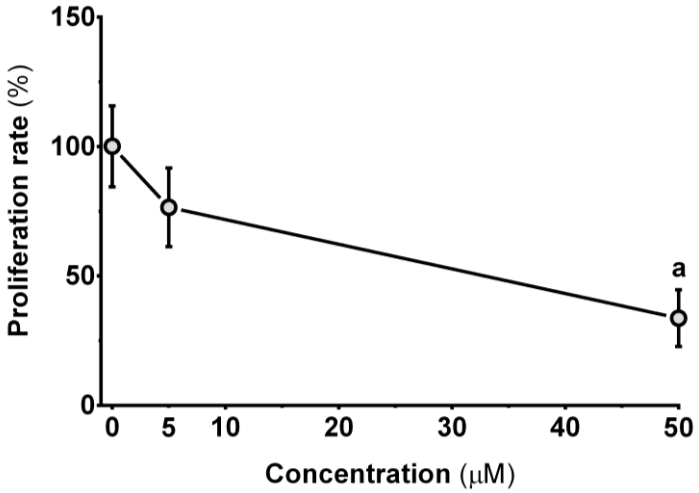


Figure 3.8 Effect of epigallocatechin gallate (EGCG; 5 and 50 µM) in the proliferation of human Sertoli cells (hSCs). The figure shows pooled data of independent experiments. hSCs growth is presented as percentage and control value was set at 100%. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to control.

Glucose consumption was stimulated by exposure of hSCs to 50 µM of EGCG

We have firstly evaluated glucose uptake as it is the main metabolic substrate of cultured hSCs. Our results showed a glucose consumption of 49 ± 16 and 47 ± 13 pmol/µg protein in non-exposed hSCs and those exposed to 5 µM of EGCG, respectively (Table 3.3). There was a high increase in glucose consumption in hSCs exposed to 50 µM of EGCG (161 ± 47 pmol/µg protein), relative to cells from the other experimental groups (Table 3.3). Glucose transport through the hSCs cytoplasmic membrane is mediated by glucose transporters (GLUTs), particularly GLUT1, GLUT2 and GLUT3 [22]. The protein levels of GLUT1 and GLUT3 (Figure 3.9A) were not altered in hSCs after exposure to EGCG. However, there was a decrease in GLUT2 protein levels in hSCs exposed to 50 µM of EGCG (0.56 ± 0.13-fold variation to the control) relative to non-exposed hSCs and those exposed to 5 µM of EGCG (1.15 ± 0.13-fold variation to the control) (Figure 3.9A).

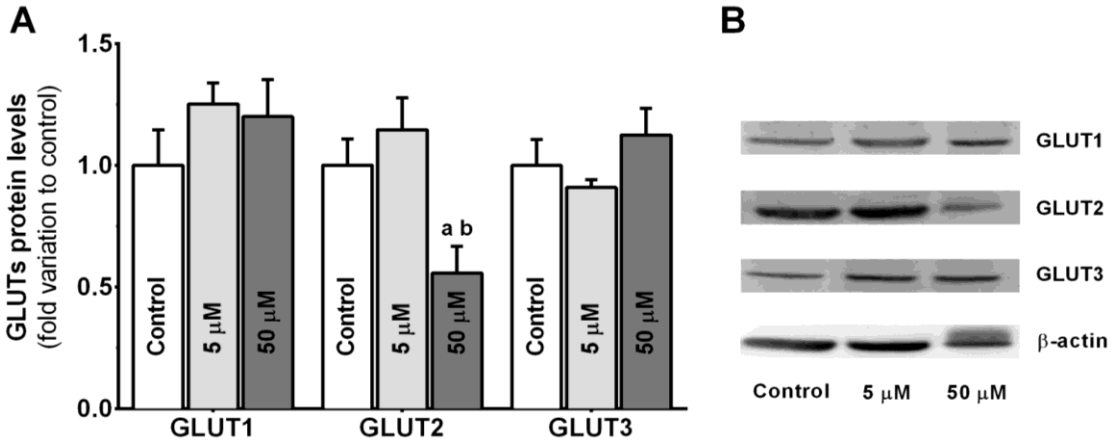


Figure 3.9 Effect of epigallocatechin gallate (EGCG; 5 and 50 µM) in glucose metabolism of human Sertoli cells. The figure shows pooled data of independent experiments, indicating glucose transporters (GLUT1, GLUT2 and GLUT3) protein levels (Panel A). Panel B displays the representative blots (of one sample) of GLUT1, GLUT2 and GLUT3. Variation in protein levels is presented as fold variation to the control. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to control; b – relative to 5 µM EGCG.

Exposure of hSCs to 50 µM of EGCG stimulated pyruvate consumption

Once glucose enters the cell, a cascade of reactions involving the enzyme phosphofructokinase 1 (PFK1), leads to the irreversible conversion of glucose to pyruvate [23]. For that reason, we evaluated PFK1 protein levels, as well as pyruvate consumption by hSCs exposed to EGCG. No differences were found in PFK1 protein levels between the experimental groups (Figure 3.10A). However, pyruvate consumption increased from 3.28 ± 0.36 pmol/µg protein in the control group to 6.25 ± 0.82 pmol/µg protein in hSCs exposed to 50 µM of EGCG (Table 3.4). Exposure of hSCs to 5 µM of EGCG did not alter pyruvate consumption (4.09 ± 0.40 pmol/µg protein) relative to control (Table 3.4).

Exposure of hSCs to 50 µM of EGCG decreased LDH and MCT4 protein levels, as well as LDH activity

In hSCs, most of the pyruvate produced is converted to lactate by LDH [5] and then exported to the intratubular fluid by the monocarboxylate transporter 4 (MCT4) [24]. Our results demonstrated a decrease in LDH protein levels in hSCs exposed to 50 µM of EGCG (0.56 ± 0.03-fold variation to the control) relative to hSCs from the control group and exposed to 5 µM of EGCG (1.08 ± 0.13-fold variation to the control) (Figure 3.10A). There was a decrease in LDH activity of hSCs exposed to 50 µM of EGCG (0.06 ± 0.02-fold variation to the control) in comparison to the control group and hSCs exposed to 5 µM of EGCG (0.91 ± 0.11-fold variation to the control) (Figure 3.10A). Moreover, exposure of hSCs to 50 µM of EGCG also led to decreased protein levels of MCT4 (0.67 ± 0.05-fold variation to the control) comparatively to cells from the control group and those exposed to 5 µM of EGCG (1.05 ± 0.07-fold variation to the control) (Figure 3.10A). However, hSCs lactate production was not affected by exposure to EGCG (Table 3.4).

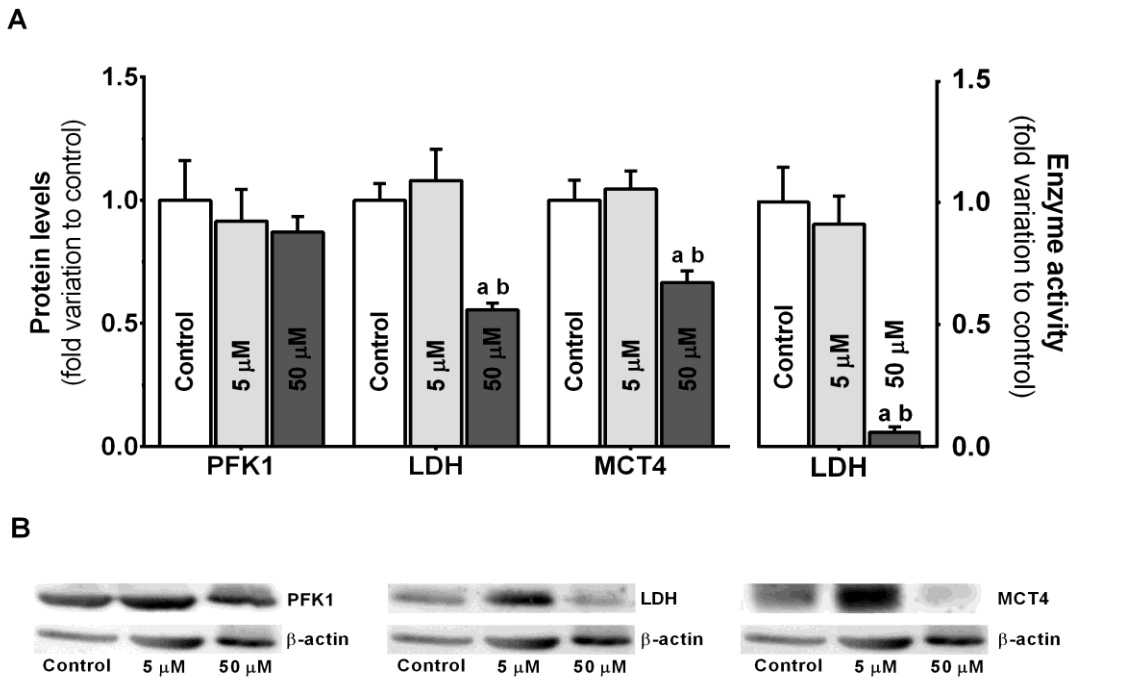


Figure 3.10 Effect of epigallocatechin gallate (EGCG; 5 and 50 µM) in pyruvate and lactate metabolism of human Sertoli cells. The figure shows pooled data of independent experiments, indicating phosphofructokinase 1 (PFK1) protein levels, lactate dehydrogenase (LDH) protein levels and activity, as well as monocarboxylate transporter 4 (MCT4) protein levels (Panel A). Panel B displays the representative blots (of one sample) of PFK1, LDH and MCT4. Variation in protein levels is presented as fold variation to the control. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to control; b – relative to 5 µM EGCG.

Alanine production was decreased in hSCs exposed to 50 µM of EGCG

Besides lactate, pyruvate can also be converted to alanine [25] or it can enter the mitochondria to originate acetyl-CoA [5]. Subsequently, acetyl-CoA can lead to citrate [26] and/or acetate production [27]. Our results showed that EGCG did not alter acetate nor citrate production by hSCs (Table 3.4). However, alanine production in hSCs exposed to 50 µM of EGCG was decreased (1.79 ± 0.50 pmol/µg protein), relative to the control group (3.13 ± 0.29 pmol/µg protein) and hSCs exposed to 5 µM of EGCG (3.74 ± 0.65 pmol/µg protein) (Table 3.4). We also evaluated the ratio lactate/alanine, which is an index of cellular redox state [22]. hSCs exposed to 50 µM of EGCG presented an increased lactate/alanine ratio (23.3 ± 0.8) when compared to the control group (10.4 ± 0.6) and hSCs exposed to 5 µM of EGCG (11.0 ± 0.4).

Table 3.4 Metabolites consumption/production and lactate/alanine ratio in human Sertoli cells from the control group and groups exposed to 5 or 50 µM of epigallocatechin gallate (EGCG).

Metabolites (pmol/µg protein)	Control	5 µM of EGCG	50 µM of EGCG
Glucose consumption	49.1 ± 16	47 ± 13	161 ± 47[a b]
Pyruvate consumption	3.28 ± 0.36	4.09 ± 0.40	6.25 ± 0.82[a]
Lactate production	43.26 ± 1.43	51.12 ± 6.70	49.69 ± 4.78
Alanine production	3.13 ± 0.29	3.74 ± 0.65	1.79 ± 0.50[a b]
Acetate production	5.08 ± 0.43	6.55 ± 1.38	5.07 ± 0.53
Citrate production	10.15 ± 0.85	10.43 ± 1.33	9.40 ± 0.56
Lactate/Alanine Ratio	10.42 ± 0.57	10.98 ± 0.36	23.26 ± 0.78[a b]

Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to control; b – relative to 5 µM of EGCG.

Mitochondrial membrane potential was decreased in hSCs exposed to 50 µM of EGCG

Mitochondrial electron transport chain (ETC) is one of the main cellular generators of ROS. ETC includes four multi-subunit complexes (complexes I–IV), responsible for oxidative phosphorylation [28]. The electron transport generates an electrochemical proton gradient across the inner mitochondrial membrane, measured as mitochondrial membrane potential, which drives ATP synthesis by complex V [29]. Our results did not show alterations in the protein levels of mitochondrial complexes I-V in hSCs exposed to EGCG (Table 3.5). However, JC1 ratio decreased after hSCs exposure to the highest dose of EGCG (50 µM) (1.05 ± 0.07) relative to control group (1.85 ± 0.14) and hSCs exposed to 5 µM of EGCG (1.80 ± 0.27) (Figure 3.11A), showing a decrease in mitochondrial membrane potential in cells exposed to the highest dose of EGCG. Nevertheless, oxygen consumption was similar among the several experimental groups (Figure 3.11B).

Table 3.5 Protein expression levels of mitochondrial complexes in human Sertoli cells from the control group and groups exposed to 5 or 50 µM of epigallocatechin gallate (EGCG).

Mitochondrial Complexes	Control	5 µM of EGCG	50 µM of EGCG
CI	1.00 ± 0.01	1.05 ± 0.10	1.16 ± 0.15
CII	1.00 ± 0.01	1.05 ± 0.09	1.09 ± 0.12
CIII	1.00 ± 0.01	0.95 ± 0.03	1.00 ± 0.08
CIV	1.00 ± 0.02	1.15 ± 0.07	0.98 ± 0.09
CV	1.00 ± 0.02	0.98 ± 0.07	0.97 ± 0.06

Abbreviations: CI, NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 8 (NDUFB8); CII, succinate dehydrogenase complex, subunit B, iron sulfur (SDHB); CIII, ubiquinol-cytochrome c reductase core protein II (UQCRC2); CIV, mitochondrially encoded cytochrome c oxidase I (MTCO1); CV, ATP synthase alpha-subunit (ATP5A). Results are expressed as mean ± SEM (fold variation to the control), n = 6 for each condition.

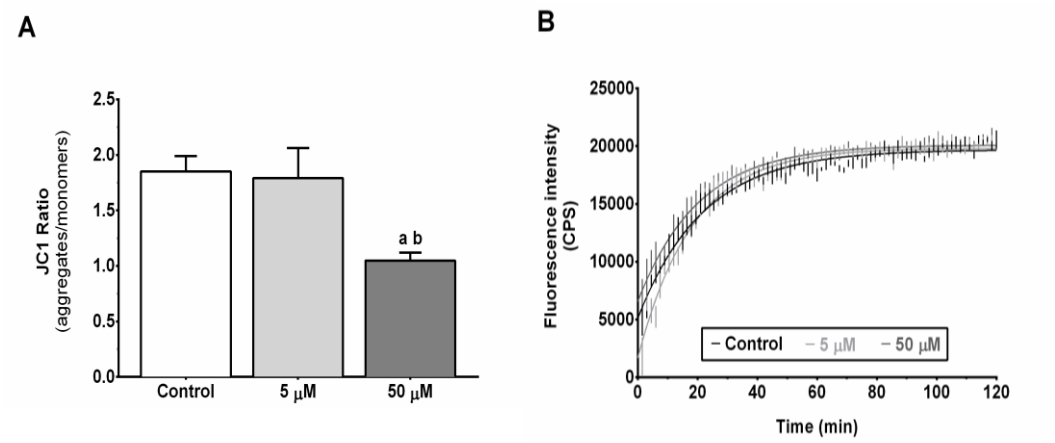


Figure 3.11 Effect of epigallocatechin gallate (EGCG; 5 and 50 µM) in mitochondrial membrane potential and extracellular oxygen consumption of human Sertoli cells. The figure shows pooled data of independent experiments, indicating JC1 ratio (Panel A) and extracellular oxygen consumption (Panel B). Results from JC1 are presented as ratio aggregates/monomers, while oxygen consumption is presented as fluorescence intensity in CPS (counts per second). Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to control; b – relative to 5 µM EGCG.

Exposure of hSCs to 50 µM of EGCG decreased protein and lipid oxidation

As high metabolic rates and mitochondrial activity are usually associated with ROS overproduction [30] and EGCG is known for its antioxidant properties [3], we evaluated its effects against oxidative damages in hSCs. We quantified the formation of end-products resultant from protein oxidation or nitration, as well as lipid peroxidation when cells were exposed to 5 or 50 µM of EGCG. Our data showed decreased levels of carbonyl groups and nitration in hSCs exposed to 50 µM of EGCG (0.86 ± 0.04 and 0.80 ± 0.01-fold variation to the control, respectively) relative to cells exposed to 5 µM of EGCG (1.12 ± 0.10 and 1.08 ± 0.10-fold variation to the control, respectively) (Figure 3.12). Exposure of hSCs to 50 µM of EGCG also led to decreased levels of lipid peroxidation (0.85 ± 0.04-fold variation to the control) comparatively to hSCs from the control group and those exposed to 5 µM of EGCG (1.06 ± 0.02-fold variation to the control) (Figure 3.12).

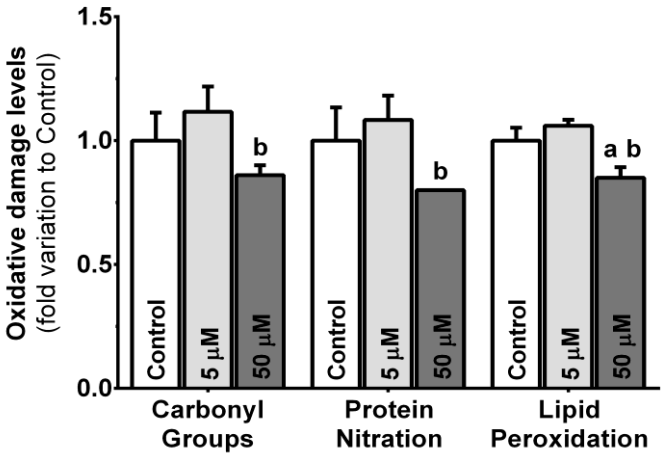


Figure 3.12 Effect of epigallocatechin gallate (EGCG; 5 and 50 µM) in oxidative damage levels of human Sertoli cells. The figure shows pooled data of independent experiments, indicating carbonyl levels, protein nitration and lipid peroxidation. Results are presented as fold variation to control. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to control; b – relative to 5 µM EGCG.

Discussion

Oxidative damage to cells and biomolecules has been associated with the pathology of several diseases/dysfunctions, including male fertility [31]. SCs are essential for the successful progression of spermatogenesis because they provide all the metabolic needs to the developing germ cells [23]. Modulation of SCs metabolism is essential for spermatogenesis [32]. Thus, modulators of SCs metabolism can be valuable to counteract male subfertility/infertility in some conditions, and tea constituents have shown promising results [9, 10]. Studies focused on the role of EGCG in male fertility are scarce and mostly focused on its effects on sperm quality [33, 34]. As male fertility capacity is highly dependent on hSCs metabolism, we evaluated the effects of EGCG on those mechanisms. Despite the intricate limitations of the *in vitro-in vivo* data extrapolations concerning metabolic studies, we consider that our *in vitro* model possesses the most important features of SCs *in vivo* and that the data obtained would be a further step in understanding the effects of EGCG on SCs physiology and function [35].

EGCG can be orally consumed in food supplements prepared from very concentrated tea extracts or in infusions (diluted). Although the bioavailability of EGCG after drinking tea is very low (0.3-0.5 µM) [14], the ingestion of 400-1200 mg of EGCG from a tea extract (fasting conditions) may result in plasma levels of 2-7 µM [15]. Thus, we selected 5 µM as the lowest concentration of EGCG in the study. As there are several studies reporting a pharmacologically relevant action of 50 µM of EGCG [16, 17], we also evaluated the effects of this concentration as well. The main objective of investigating the effects of this supraphysiologic dose of EGCG is its possible usage in a specific treatment for male reproductive dysfunctions. We choose to expose hSCs to EGCG for 24 h to mimic a chronic, prolonged and repetitive dose of EGCG in a short-time.

Most of EGCG studies report its cancer-preventive properties [36], particularly its ability to act as a potent inhibitor of cell proliferation [37]. Previous studies have evidenced metabolic similarities between hSCs and cancer cells (for review [38]). Still, studies from our research group have reported that the intensive glycolytic activity of hSCs is not always correlated with the proliferative properties of these cells [10, 39, 40], as frequently happens with cancer cells [38]. The results here presented demonstrated a dose-dependent decrease of hSCs proliferation when exposed to 5 and 50 μM of EGCG, evidencing a clear anti-proliferative effect of this compound on these testicular cells. This scenario might also be a result from EGCG cytotoxicity to hSCs. The exposure of hSCs to 50 μM of EGCG during 24 h might overcome the maximum tolerable dose, thus exerting toxicity to hSCs, which results in the observed decrease in hSCs proliferation. However, some caution should be taken when extrapolating these results to an *in vivo* situation due to the differences in the proliferating ability of Sertoli cells *in vitro* and *in vivo* [35].

EGCG has been ascribed as modulator of cells metabolism, including spermatozoa [33]. To investigate the effects of EGCG on the metabolic profile of hSCs, we selected key intervenient of the glycolytic pathway, as glucose is the main metabolic substrate of cultured hSCs and lactate is the key substrate produced by hSCs for developing germ cells [32]. Exposure of hSCs to the highest concentration of EGCG (50 μM) increased glucose consumption by these cells. Notably, a similar effect induced by 40 μM of EGCG was reported in muscle cells [41]. In hSCs, glucose uptake is mediated by GLUT1, GLUT2 and GLUT3 [22]. We have not detected any alteration in GLUT1 or GLUT3 protein levels. Though, there was a decrease in GLUT2 levels in hSCs exposed to 50 μM of EGCG. This suggests that the increased glucose uptake observed was sustained by GLUT1 and GLUT3, which are described to have a higher affinity for glucose than GLUT2, allowing cells to withstand a high rate of glucose transport [42]. As GLUT2 presents a high Michaelis constant (K_m), its rate of glucose uptake decreases in parallel with the decrease of glucose concentration under the physiological range [43]. Thus, the decrease in GLUT2 protein levels, can be a result of an adaptive response to the decrease in glucose concentration related to the consume of glucose present in the culture media [44].

Once inside the cell, glucose is readily converted to pyruvate, in a process mediated by PFK1, which is a control point of the glycolytic pathway [45]. The protein levels of this enzyme in hSCs were not altered by exposure to EGCG, suggesting that pyruvate production is not compromised. In hSCs, the great majority of pyruvate is transformed into lactate, by LDH [5], and then transported to the extracellular compartment by MCT4, where it can be used by developing germ cells [23]. Exposure of hSCs to 50 μM of EGCG led to a decrease in LDH protein levels and activity, and to decreased MCT4 protein levels. Nevertheless, no differences were found in lactate production by hSCs exposed to this concentration of EGCG when compared with the other conditions. These results suggest that as lactate is being produced in normal amounts, LDH and MCT4 protein and/or enzyme activity levels are not rate limiting.

The maintenance of lactate production at control levels by hSCs exposed to 50 μM of EGCG is essentially due to the differences in pyruvate metabolism. Besides the higher pyruvate consumption, we also verified a decrease in alanine production in these cells. As alanine is attained from pyruvate in a reversible reaction catalyzed by alanine transaminase (ALT) [46] and its production is decreased in hSCs exposed to EGCG (50 μM), our results suggest that the higher pyruvate consumption is also responsible for the maintenance of the normal production of lactate. This scenario resulted in the increase of the lactate/alanine ratio in those cells. Lactate/alanine ratio is linked to the intracellular redox status, as it reflects the nicotinamide adenine dinucleotide reduced/oxidized ($NADH/NAD^+$) ratio, which in turn is directly implicated in energy metabolism. It constitutes a metabolic node well suited for integration of energy metabolism and an optimal $NADH/NAD^+$ ratio is essential for normal mitochondrial function [47].

The maintenance of pyruvate production by the glycolytic pathway under certain levels is also crucial to maintain a proper mitochondrial function, as it enters the mitochondria to be converted in acetyl-CoA [48]. Then, acetyl-CoA can enter the Krebs cycle, where it is converted to citrate [26], or it can be exported to the cytosol and form acetate [27] for fatty acids and cholesterol synthesis [49]. When there is a Krebs cycle truncation, citrate can also be transported to the cytosol or to extracellular compartment [26]. Once no alterations were found either in acetate or citrate extracellular production by hSCs after exposure to EGCG, we suggest that pyruvate is not only being used to sustain lactate production, but also to fuel the Krebs cycle. These results support that the functionality of hSCs Krebs cycle is not being compromised by exposure to EGCG. The normal oxygen consumption among the experimental groups corroborates our data. In fact, if oxygen consumption was increased, oxidative metabolism should be occurring towards CO_2 production. As oxygen consumption was not affected by exposure to EGCG and neither acetate nor citrate are being exported to the extracellular compartment, these two metabolites should be used for lipid synthesis within the cytosol. This may be part of the additional nutritional support of spermatogenesis. However, we detected a decrease in mitochondrial membrane potential of hSCs exposed to 50 μM of EGCG. The same result was observed in a previous study where hepatocytes were exposed to 30 μM of EGCG [50]. Still, no differences were found in the protein levels of mitochondrial complexes I-V, which are responsible for oxidative phosphorylation (OXPHOS) and ATP synthesis [28]. Despite protein levels of mitochondrial complexes were not altered, EGCG may have some inhibitory effects on the activity of those complexes, as it was previously described [51, 52]. This may result in a mitochondrial uncoupling, as reported [53], which may also be related to the cytotoxic effect of this dose. Although Krebs cycle homeostasis is not being compromised by exposure of hSCs to EGCG, we observed a dissipation of the mitochondrial potential, which might be associated with a reprogramming of the cellular metabolic pathways to sustain hSCs substrate requirements [54]. On the other hand, it is known that OXPHOS in mitochondria involves ROS production [28] and that EGCG can be accumulated in mitochondria modulating

OS [55]. In fact, there was a decrease in protein oxidation and nitration, as well as in lipid peroxidation in hSCs exposed to 50 μM of EGCG. This corroborates the protective role attributed to high doses of EGCG against ROS overproduction [55]. Hence, EGCG seems to have a dual effect in hSCs mitochondrial function: while it decreases hSCs mitochondrial functioning, it also protects hSCs from ROS-induced damages due to its potent antioxidant potential.

In conclusion, our study demonstrates a dose-dependent modulating action of EGCG in hSCs metabolism, mitochondrial functionality and oxidative profile. EGCG at high concentration (50 μM) could modulate hSCs metabolism, maintaining lactate production and Krebs cycle functionality. Despite 50 μM of EGCG might decrease ETC function, Krebs cycle was preserved. Additionally, our data suggest that citrate and acetate are being used for lipid synthesis within the cytosol, which might be part of an additional nutritional support for spermatogenesis. Moreover, 50 μM of EGCG was able to decrease oxidative damage to proteins and lipids. This may be of extreme importance for the improvement of spermatogenesis and male fertility because OS is on the basis of several fertility problems [56]. The strong enhancement of glucose uptake verified in hSCs exposed to EGCG supports previously reported benefits of EGCG against diabetes *mellitus* [57, 58], which is characterized by glucose intolerance [59] and strongly affects hSCs function [5]. Although the dose of 50 μM of EGCG might not be physiologically attained by ingestion of EGCG-rich products, it could be attained with a local administration, supplements or certain medications. Thus, the main objective using this supraphysiologic dose was indeed to test the possible usage of EGCG in a specific treatment for male reproductive dysfunctions. Besides, the bioavailability of EGCG to SCs *in vivo* should be further investigated. Herein, EGCG is proposed as a novel modulator of cultured hSCs metabolic and oxidative profiles, which may have important effects in the nutritional support of spermatogenesis, particularly under unfavorable conditions, such as those evidenced by men with metabolic diseases.

References

1. Dias TR, et al. (2014) White tea as a promising antioxidant medium additive for sperm storage at room temperature: a comparative study with green tea. Journal of agricultural and food chemistry 62(3):608-17.
2. Khan N and Mukhtar H (2007) Tea polyphenols for health promotion. Life sciences 81(7):519-33.
3. Lombardo F, et al. (2011) The role of antioxidant therapy in the treatment of male infertility: an overview. Asian Journal of Andrology 13(5):690-97.
4. Dias TR, et al. (2014) Natural Products as Modulators of Spermatogenesis: The Search for a Male Contraceptive. Current molecular pharmacology 7(2):154-66.
5. Dias TR, et al. (2014) Sperm glucose transport and metabolism in diabetic individuals. Molecular and cellular endocrinology 396(1):37-45.
6. Alves MG, et al. (2014) Metabolic Cooperation in Testis as a Pharmacological Target: From Disease to Contraception. Current molecular pharmacology 7(2):83-95.
7. Dias TR, et al. (2016) White tea intake prevents prediabetes-induced metabolic dysfunctions in testis and epididymis preserving sperm quality. The Journal of Nutritional Biochemistry 37:83-93.
8. Oliveira PF, et al. (2015) White tea consumption restores sperm quality in prediabetic rats preventing testicular oxidative damage. Reproductive BioMedicine Online 31(4):544-56.
9. Martins AD, et al. (2014) Effect of white tea (Camellia sinensis (L.)) extract in the glycolytic profile of Sertoli cell. European journal of nutrition 53(6):1383-91.

10. Dias TR, et al. (2015) Dehydroepiandrosterone and 7-oxo-dehydroepiandrosterone in male reproductive health: implications of differential regulation of human Sertoli cells metabolic profile. The Journal of Steroid Biochemistry and Molecular Biology 154:1-11.
11. Oliveira PF, et al. (2009) Intracellular pH regulation in human Sertoli cells: role of membrane transporters. Reproduction 137(2):353-59.
12. Ivell R (2007) Lifestyle impact and the biology of the human scrotum. Reproductive Biology and Endocrinology 5(15):1477-82.
13. Alves MG, et al. (2012) In vitro cultured human Sertoli cells secrete high amounts of acetate that is stimulated by 17β-estradiol and suppressed by insulin deprivation. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1823(8):1389-94.
14. Yang CS, et al. (2009) Cancer prevention by tea: animal studies, molecular mechanisms and human relevance. Nature Reviews Cancer 9(6):429-39.
15. Chow HS, et al. (2003) Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals. Clinical Cancer Research 9(9):3312-19.
16. Albrecht DS, et al. (2008) Epigallocatechin-3-gallate (EGCG) inhibits PC-3 prostate cancer cell proliferation via MEK-independent ERK1/2 activation. Chemico-biological interactions 171(1):89-95.
17. Weber A-A, et al. (2004) Mechanisms of the inhibitory effects of epigallocatechin-3 gallate on platelet-derived growth factor-BB-induced cell signaling and mitogenesis. FASEB Journal 18(1):128-30.
18. Fricker S (1994) The application of sulforhodamine B as a colorimetric endpoint in a cytotoxicity assay. Toxicology in vitro 8(4):821-22.
19. Alves MG, et al. (2011) Substrate selection in hearts subjected to ischemia/reperfusion: role of cardioplegic solutions and gender. NMR in Biomedicine 24(9):1029-37.
20. Dias TR, et al. (2013) Insulin deprivation decreases caspase-dependent apoptotic signaling in cultured rat Sertoli cells. ISRN Urology 2013:970370.
21. Salvioli S, et al. (1997) JC-1, but not DiOC 6 (3) or rhodamine 123, is a reliable fluorescent probe to assess ΔΨ changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS letters 411(1):77-82.
22. Oliveira PF, et al. (2012) Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells. Biochim Biophys Acta (BBA) 1820(2):84-89.
23. Martins AD, et al. (2013) Control of Sertoli cell metabolism by sex steroid hormones is mediated through modulation in glycolysis-related transporters and enzymes. Cell and tissue research 354(3):861-68.
24. Galardo MN, et al. (2007) The AMP-activated protein kinase activator, 5-aminoimidazole-4-carboxamide-1-bD-ribonucleoside, regulates lactate production in rat Sertoli cells. Journal of molecular endocrinology 39(4):279-88.
25. Kaiser GR, et al. (2005) Metabolism of amino acids by cultured rat Sertoli cells. Metabolism 54(4):515-21.
26. Costello LC and Franklin RB (2006) The clinical relevance of the metabolism of prostate cancer; zinc and tumor suppression: connecting the dots. Molecular cancer 5(1):17.
27. Yamashita H, et al. (2006) Acetate generation in rat liver mitochondria; acetyl-CoA hydrolase activity is demonstrated by 3-ketoacyl-CoA thiolase. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 1761(1):17-23.
28. Turrens JF (2003) Mitochondrial formation of reactive oxygen species. The Journal of physiology 552(2):335-44.
29. Lu H and Cao X (2008) GRIM-19 is essential for maintenance of mitochondrial membrane potential. Molecular biology of the cell 19(5):1893-902.
30. Aitken RJ, et al. (2010) Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. Human Reproduction 25(10):2415-26.
31. Makker K, et al. (2009) Oxidative stress & male infertility. The Indian Journal of Medical Research 129(4):357-67.
32. Rato L, et al. (2012) Metabolic regulation is important for spermatogenesis. Nature Reviews Urology 9(6):330-38.
33. De Amicis F, et al. (2012) Epigallocatechin gallate affects survival and metabolism of human sperm. Molecular nutrition and food research 56(11):1655-64.
34. Dias TR, et al. (2016) The single and synergistic effects of the major tea components caffeine, epigallocatechin-3-gallate and l-theanine on rat sperm viability. Food & function 7(3):1301-05.
35. Reis M, et al. (2015) Sertoli cell as a model in male reproductive toxicology: Advantages and disadvantages. Journal of Applied Toxicology 35(8):870-83.
36. Azam S, et al. (2004) Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. Toxicology in vitro 18(5):555-61.
37. Kang HW (2015) Inhibitory Effect of Cancer Cells Proliferation from Epigallocatechin-3-O-gallate. Journal of Food and Nutrition Research 3(4):281-84.
38. Oliveira PF, et al. (2015) The Warburg effect revisited—lesson from the Sertoli cell. Medicinal research reviews 35(1):126-51.

39. Martins AD, et al. (2015) Leptin modulates human Sertoli cells acetate production and glycolytic profile: a novel mechanism of obesity-induced male infertility? Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1852(9):1824-32.
40. Jesus TT, et al. (2015) Mammalian target of rapamycin controls glucose consumption and redox balance in human Sertoli cells. Fertility and sterility 105(3):825-33.
41. Zhang ZF, et al. (2010) Epigallocatechin-3-O-gallate (EGCG) protects the insulin sensitivity in rat L6 muscle cells exposed to dexamethasone condition. Phytomedicine 17(1):14-18.
42. Wood IS and Trayhurn P (2003) Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. British Journal of Nutrition 89(01):3-9.
43. Eny KM, et al. (2008) Genetic variant in the glucose transporter type 2 is associated with higher intakes of sugars in two distinct populations. Physiological genomics 33(3):355-60.
44. Macheda ML, et al. (2005) Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. Journal of cellular physiology 202(3):654-62.
45. Rato L, et al. (2013) High-energy diets may induce a pre-diabetic state altering testicular glycolytic metabolic profile and male reproductive parameters. Andrology 1(3):495-504.
46. Miyashita Y, et al. (2007) Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in Arabidopsis thaliana. The Plant Journal 49(6):1108-21.
47. Alves M, et al. (2013) Exposure to 2, 4-dichlorophenoxyacetic acid alters glucose metabolism in immature rat Sertoli cells. Reproductive Toxicology 38:81-88.
48. Kim K-H (1997) Regulation of mammalian acetyl-coenzyme A carboxylase. Annual review of nutrition 17(1):77-99.
49. Shimazu T, et al. (2010) Acetate metabolism and aging: an emerging connection. Mechanisms of ageing and development 131(7):511-16.
50. Kucera O, et al. (2015) In Vitro Toxicity of Epigallocatechin Gallate in Rat Liver Mitochondria and Hepatocytes. Oxidative Medicine and Cellular Longevity 2015.
51. Valenti D, et al. (2013) Negative modulation of mitochondrial oxidative phosphorylation by epigallocatechin-3 gallate leads to growth arrest and apoptosis in human malignant pleural mesothelioma cells. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1832(12):2085-96.
52. Zheng J and Ramirez VD (2000) Inhibition of mitochondrial proton F0F1-ATPase/ATP synthase by polyphenolic phytochemicals. British journal of pharmacology 130(5):1115-23.
53. Lee M-S and Kim Y (2009) (−)-Epigallocatechin-3-gallate Enhances Uncoupling Protein 2 Gene Expression in 3T3-L1 Adipocytes. Bioscience, biotechnology, and biochemistry 73(2):434-36.
54. Samudio I, et al. (2009) Mitochondrial uncoupling and the Warburg effect: molecular basis for the reprogramming of cancer cell metabolism. Cancer research 69(6):2163-66.
55. Schroeder EK, et al. (2009) Green tea epigallocatechin 3-gallate accumulates in mitochondria and displays a selective antiapoptotic effect against inducers of mitochondrial oxidative stress in neurons. Antioxidants and Redox Signaling 11(3):469-80.
56. Agarwal A, et al. (2008) Clinical Relevance of Oxidative Stress in Male Factor Infertility: An Update. American Journal of Reproductive Immunology 59(1):2-11.
57. Ortsater H, et al. (2012) Diet supplementation with green tea extract epigallocatechin gallate prevents progression to glucose intolerance in db/db mice. Nutrition and Metabolism 9:11.
58. Chen N, et al. (2009) Green tea, black tea, and epigallocatechin modify body composition, improve glucose tolerance, and differentially alter metabolic gene expression in rats fed a high-fat diet. Nutrition Research 29(11):784-93.
59. Rato L, et al. (2015) Testicular Metabolic Reprogramming in Neonatal Streptozotocin-Induced Type 2 Diabetic Rats Impairs Glycolytic Flux and Promotes Glycogen Synthesis. Journal of Diabetes Research 2015:973142.

L-theanine promotes cultured human Sertoli cells proliferation and modulates glucose metabolism

Abstract

L-theanine is the major free amino acid present in tea (*Camellia sinensis* L.). The effects of several tea constituents on male reproduction have been investigated, but L-theanine has been overlooked. Sertoli cells (SCs) are essential for the physical and nutritional support of germ cells. In this study, we aimed to investigate the ability of L-theanine to modulate important mechanisms of human SCs (hSCs) metabolism, mitochondrial function and oxidative profile, which are essential to prevent or counteract spermatogenesis disruption in several health conditions. We evaluated the effect of a dose of L-theanine attained by tea intake (5 μM) or a pharmacological dose (50 μM) on the metabolism (proton nuclear magnetic resonance and Western blot), mitochondrial functionality (protein expression of mitochondrial complexes and JC1 ratio) and oxidative profile (carbonyl levels, nitration and lipid peroxidation) of cultured human Sertoli cells (hSCs). Exposure of hSCs to 50 μM of L-theanine increased cell proliferation and glucose consumption. In response to this metabolic adaptation, there was an increase in mitochondrial membrane potential, which may compromise the prooxidant-antioxidant balance. Still, no alterations were observed regarding the oxidative damages. A pharmacological dose of L-theanine (50 μM) prompts an increase in hSCs proliferation and a higher glucose metabolization to sustain the pool of Krebs cycle intermediates, which are crucial for cellular bioenergetics and biosynthesis. This study suggests an interplay between glycolysis and glutaminolysis in the regulation of hSCs metabolism.

Keywords: antioxidant; cell metabolism; glutamate; L-theanine; mitochondria; Sertoli cell.

Introduction

Tea (*Camellia sinensis* L.) consumption has been associated with a wide variety of health benefits, thus encouraging many people to include it in their daily alimentary routine. Although tea composition is highly variable according to the type (white, green, oolong or black) and geographical origin, the most bioactive tea compounds include methylxanthines, catechins and amino acids. L-theanine (L-γ-glutamylethylamide) is a nonproteinogenic amino acid almost exclusively found in this botanical species. L-theanine is usually associated with tea unique taste and relaxation properties. For that reason, it gained popularity and became a common ingredient in functional beverages and food supplements.

Human male reproduction is highly reliant on the success of sperm production. This complex process known as spermatogenesis is mainly sustained by Sertoli cells (SCs), which are the somatic cells present in the seminiferous tubules of the testes. These cells constitute the blood-

testis-barrier (BTB), which is crucial for the physical support and protection of developing germ cells. They are also responsible for the production of several growth factors and nutrients, essential for germ cells survival and differentiation into spermatozoa [1]. The normal function of SCs metabolic processes is crucial for the preservation of male reproductive potential. These cells produce high amounts of lactate, which is then used as energy source by developing germ cells. The main substrate used by SCs to produce lactate is glucose, but they can also metabolize amino acids such as glutamine.

Several pathological conditions, such as diabetes *mellitus* and obesity, may trigger severe alterations in SCs metabolism, thus disturbing spermatogenesis and compromising male fertility. In fact, the increasing incidence of those diseases around the world is being accompanied by a decrease in male reproductive health. This scenario highlighted the need to find new modulators of SCs metabolism to prevent the negative effects of those diseases on male reproduction. Tea and its constituents have previously shown the ability to modulate SCs function *in vitro* [2-4] and improve male reproductive potential *in vivo* [5]. One of the major issues is that the high metabolic rates of SCs are associated with the production of reactive oxygen species (ROS) and if something disturbs the prooxidant-antioxidant homeostasis, the function of these testicular cells may be compromised. As L-theanine has demonstrated a preventive role against oxidative stress (OS) in other cell types, we hypothesized that this tea amino acid could maintain the redox homeostasis in human SCs (hSCs) preserving its function. Hence, we aimed to investigate the ability of L-theanine to modulate important mechanisms of hSCs metabolism, mitochondrial function and oxidative profile, which are known to be essential to prevent or counteract spermatogenesis disruption in several health conditions.

Materials and Methods

Chemicals

Fetal bovine serum (FBS; S0615): Biochrom (Leonorenstr, Berlin, Germany); Insulin–transferrin–sodium selenite (ITS; 41400045): Gibco™ by Life Technologies (Carlsbad, California, USA); L-theanine (ab141187; CAS number 3081-61-6) and Extracellular O_2 consumption assay (ab197243): Abcam (Cambridge, UK); Tris-base (MB01601) NZYTech (Lisbon, Portugal); Mammalian protein extraction reagent (M-PER) (78501) and lactate dehydrogenase (LDH) enzymatic assay kit (88954): Thermo Scientific (Waltham, MA, USA); 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC1; T3168): Molecular Probes (Eugene, OR, USA); All other chemicals: Sigma-Aldrich (St. Louis, MO, USA) unless specifically stated.

Cell primary cultures and experimental groups

The processing of human testicular tissue was performed at Centre for Reproductive Genetics Professor Alberto Barros (Porto, Portugal) according to local, national, and European ethical committees' guidelines and the Declaration of Helsinki. Six testicular biopsies were obtained

from six different patients with conserved spermatogenesis, after informed written consent. hSCs were obtained from the cells left in tissue culture plates after each patient's treatment. Cells from each individual were plated separately in six Cell+ culture flasks (Sarstedt, Nümbrecht, Germany) and incubated at 33-34°C, in a controlled atmosphere of 5% CO_2. Sertoli cell culture purity was assessed by the immunoperoxidase technique. Briefly, cells were incubated with primary polyclonal antibody and processed by the labelled streptavidin–biotin method using an ExtrAvidin-Peroxidase Staining Kit (Sigma-Aldrich). Besides, specific protein markers, the anti-Mullerian hormone and Vimentin, were used to assess the purity of hSCs cultures [6]. After 96 h, cultures were examined by phase contrast microscopy and only hSCs with other cell contaminants below 5% were used.

Cells were left to grow until reach 90-95% confluence and cells from each flask were divided into three culture plates (Bioportugal, Porto, Portugal), making a total of 18 plates. After reaching 90-95% confluence, the culture media of the culture plates was replaced by serum-free media (Dulbecco's modified eagle medium:Ham's F12 nutrient mixture (DMEM:F12) supplemented with ITS medium (10 µg/mL insulin – 5.5 µg/mL transferrin – 0.0067 µg/mL sodium selenite). Three experimental groups were defined: a control group (without L-theanine) and two groups supplemented either with 5 or 50 µM of L-theanine. Previous studies reported that the ingestion of 25-100 mg of L-theanine (either as tea or capsules) correspond to a concentration of 5 - 25 µM in human plasma [7]. However, the beneficial health effects of L-theanine are mostly ascribed to higher doses of 200 mg, which correspond to a bioavailability of 50 µM. Thus, we decided to use 5 and 50 µM in this study. Besides, this will also allow the comparison with previously studied tea components, epigallocatechin gallate (EGCG) and caffeine, using this *in vitro* model [2, 3]. After 24 h of treatment with 0 (no L-theanine), 5 or 50 µM of L-theanine, cells and the respective culture media were collected. This time point was defined based on the fact that in serum, the concentration of L-theanine starts to drop slowly within 24 h [7]. Trypan Blue Exclusion test was performed to guarantee cellular integrity after the 24 h treatment, which averaged 85–90%.

Sulforhodamine B assay

A sulforhodamine B (SRB) colorimetric assay was performed as previously described [3], to evaluate hSCs proliferative responses to the 24 h exposure to 0, 5 or 50 µM of L-theanine. A blank was prepared with Tris base (pH 10) and the absorbance was read at 492 nm. Absorbance readings of SRB-stained cells give a direct measure of cell numbers. To obtain concentration-response curves we defined the cell growth of the control group as 100% and calculated the cell growth of treated groups relative to control.

MTT reduction

To evaluate hSCs metabolic viability after exposure to L-theanine we have measured the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a purple

formazan product. In brief, hSCs were cultured in a 96-well culture plate with DMEM:F12 supplemented with 10% FBS. After reaching 90-95% confluence, hSCs were cultured with ITS medium supplemented with 0, 5 or 50 μM of L-theanine for 24 h. Then, the medium was removed and replaced by 150 μL of freshly prepared ITS medium. MTT was firstly dissolved in warm phosphate-buffered saline (PBS) 1x (5 mg/mL) and protected from the light. MTT solution (15 μL) was added to each well to attain a final concentration of 0.5 mg/mL per well and cells were incubated during 3 h at 37°C. At the end of incubation, the media were removed, and formazan crystals were dissolved in 100 μL of dimethyl sulfoxide (DMSO) by gentle shaking for 10 min at room temperature (RT). A blank with DMSO was made for normalization. Absorbance was recorded at 570 nm to quantify formazan formation (directly proportional to the number of viable cells), and at 655 nm for reference. hSCs metabolic viability was expressed in fold variation to control.

LDH leakage and intracellular enzymatic activity

To evaluate possible cytotoxicity of L-theanine to hSCs we assessed LDH leakage (from damaged or destroyed cells) into the extracellular fluid and intracellular LDH activity levels after 24 h of exposure to the experimental doses. LDH levels were spectrophotometrically determined using the LDH enzymatic assay kit according to the manufacturer's instructions. For LDH leakage, 50 μL of extracellular medium were mixed with 50 μL of LDH assay substrate and incubated at RT for 30 min. Then, 50 μL of LDH stop solution were added to stop the enzymatic activity and absorbance was measured at 490 nm and 630 nm using an Anthos 2010 microplate reader (Biochrom, Berlin, Germany). Results were normalized to the blank and expressed as fold variation to control group. LDH intracellular activity was evaluated as previously described [2]. LDH enzymatic activities were calculated as units per milligram of protein using the molar extinction factor (ε) and final expressed as fold variation to the control group.

Autophagy

hSCS were grown in DMEM:F12 supplemented with 10% FBS, in 96-well plates until reach 80-90% confluence. After treatment with 0, 5 or 50 μM of L-theanine for 24 h, the media were removed, and cells were washed with PBS. Then, cells were incubated with 100 μL of propidium iodide (1 μg/mL in PBS) at RT for 2 min. Cells were washed again with PBS and incubated with 100 μL of dansylcadaverine (1 μg/mL in PBS) at 37°C for 10 min. Afterwards, cells were washed with PBS and incubated with 100 μL of Hoechst (10 μg/mL in PBS) at RT, for 10 min. During the incubation times, the 96-well plate was protected from light to avoid loss of fluorescence. After washing again, fresh PBS was added to each well and fluorescence was immediately analyzed using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, USA) pre-heated at 37°C. Death cells stained with propidium iodide were detected at 530 ± 25/590 ± 35 nm (excitation/emission), autophagic cells stained with dansylcadaverine were detected at 360 ± 40/528 ± 20 nm (excitation/emission) and cell nucleus stained with Hoechst were detected at 360 ± 40/460 ± 40 (excitation/emission). Results were normalized to the number of cells and

expressed as fold variation to the control group. A positive control with DMSO 10% was used for test validity.

Proton nuclear magnetic resonance spectroscopy

Culture medium was collected before hSCs treatment (0 h) and after the 24 h of treatment with L-theanine, to allow the analysis of metabolites production/consumption during that incubation period. 1H-NMR spectra of hSCs extracellular culture media were acquired and quantified using the previous described conditions [5]. Sodium fumarate (final concentration of 1 mM) was used as internal reference (6.50 ppm) to quantify the following metabolites present in hSCs extracellular media (multiplet, ppm): lactate (doublet, 1.33); alanine (doublet, 1.45) and H1-α-glucose (doublet, 5.22). Relative areas of ^{1}H-NMR resonances and metabolites concentrations were quantified as described [5]. Results are presented as metabolite consumption or production in pmol/cell.

Western blot

Total protein fraction from hSCs was isolated using M-PER according to manufacturer's instructions. Proteins were fractionated in 12% polyacrylamide gels, then the separated proteins were transferred to previously activated polyvinylidene difluoride (PVDF) membranes and blocked for 90 min with a 5% non-fat milk solution at RT. Afterwards, the membranes were incubated overnight (4°C) with rabbit anti-monocarboxylate transporter 4 (MCT4) (1:1000, sc-50329, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-LDH (1:10000, ab52488, Abcam, Cambridge, UK) or mouse total OXPHOS cocktail (1:1000, ab110413, Abcam, Cambridge, UK) primary antibodies. Mouse anti-ß-actin (1:5000, MA5-15739, Thermo Scientific, Waltham, MA, USA) was used as the protein loading control. The immunoreactive proteins were detected separately and visualized after incubation (90 min at RT) with the respective secondary antibodies: goat anti-rabbit IgG-alkaline phosphatase (AP) (1:5000, A3687) or goat anti-mouse IgG-AP (1:5000, A3562). Membranes were reacted with ECF™ (GE, Healthcare, Buckinghamshire, UK) and read with the BioRad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK). Densities from each band were obtained with BIO-PROFIL Bio-1D Software from Quantity One (Vilber Lourmat, Marne-la-Vallée, France) according to standard methods. The band density attained was divided by the corresponding ß-actin band intensities and expressed as fold variation (induction/reduction) relative to the control group.

Mitochondrial membrane potential

The fluorescent probe JC1 was used to measure the mitochondrial membrane potential of hSCs after exposure to 0, 5 or 50 µM of L-theanine, as previously described [3]. The accumulation of the JC1 dye in mitochondria depends upon mitochondrial membrane potential. Fluorescence intensities were analyzed immediately using a Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek, Winooski, USA) pre-heated at 37°C. Cells with functional mitochondria exhibited JC1 aggregates that were detected at 550/590nm (excitation/emission), while cells with

dysfunctional mitochondria mainly exhibited JC1 monomers detected at 485/535nm (excitation/emission). The JC1 ratio (aggregates/monomers) was calculated for each condition as a measure of mitochondrial functionality. Results are presented as fold variation to the control group. A positive control with 10 μM of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) was used for assay validation.

Extracellular oxygen consumption assay

Oxygen consumption is one of the most informative and direct measures of mitochondrial function. We used the extracellular oxygen consumption assay kit (ab197243; Abcam, Cambridge, UK) as previously described [3], to measure the respiration of hSCs after 24 h exposure to 0, 5 or 50 μM of L-theanine. A positive control of sodium bisulfite was used for assay validation. Fluorescence intensities were analyzed immediately using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, USA) pre-heated at 37°C. Extracellular oxygen consumption of hSCs was measured at 1.5 min intervals for 120 min at 380/650nm (excitation/emission). Cells respiration leads to oxygen depletion from the surrounding environment, resulting in the increase in fluorescence signal. Fluorescence intensities were normalized to the blank and expressed as counts per second (CPS) *versus* time (min).

Analysis of carbonyl groups, nitration and lipid peroxidation

Carbonyl groups, nitration and lipid peroxidation are usually used as biomarkers for oxidation and can be evaluated by measuring its resulting products, 2,4-dinitrophenol (DNP), nitro-tyrosine and 4-hydroxynonenal (4-HNE), respectively. The content of these adducts in hSCs after exposure to L-theanine was evaluated by slot-blot technique. The resulting PVDF membranes were incubated overnight (4°C) with rabbit anti-DNP (D9656, Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-nitro-tyrosine (#9691, Cell signaling Technology, Leiden, Netherlands) or goat anti-4-HNE (AB5605, Merck Millipore Temecula, USA) primary antibodies (dilution 1:5000). The immunoreactive proteins were detected separately and visualized with goat anti-rabbit IgG-AP (1:5000, A3687) or rabbit anti-goat IgG-AP (1:5000, A4187). Results were expressed as fold variation to the control group.

Statistical analysis

Statistical significance was assessed by one-way ANOVA, followed by Dunn post-test, using the GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). All data are presented as mean ± SEM (n = 6). Differences with $p < 0.05$ were considered statistically significant.

Results

Pharmacological dose of L-theanine induces an increase in hSCs proliferation while maintaining cellular metabolic viability

Our results showed an increase in hSCs proliferation after exposure to a pharmacological dose of 50 µM of L-theanine (191 ± 17%), not only relative to the control group (100 ± 8%) but also to the group of hSCs exposed to 5 µM of L-theanine (121 ± 7%) (Figure 3.13A). Nonetheless, hSCs metabolic viability, as reflected by the MTT assay, was maintained when cultured with 5 or 50 µM of L-theanine (1.09 ± 0.14 and 1.02 ± 0.18-fold variation to control, respectively) in comparison to the non-exposed hSCs (Figure 3.13B). The addition of 5 or 50 µM of L-theanine to the culture medium was not cytotoxic to hSCs, as demonstrated by the LDH leakage assay (Figure 3.13C). The group of hSCs exposed to 5 µM of L-theanine presented a LDH leakage of 1.23 ± 0.24-fold variation to control, a similar value to those exposed to 50 µM of L-theanine (1.27 ± 0.17-fold variation to control) and the control group (without L-theanine). Moreover, an autophagy assay was performed to evaluate if the selected doses of L-theanine could affect hSCs autophagic process, but no differences were found among the experimental groups (Figure 3.13D).

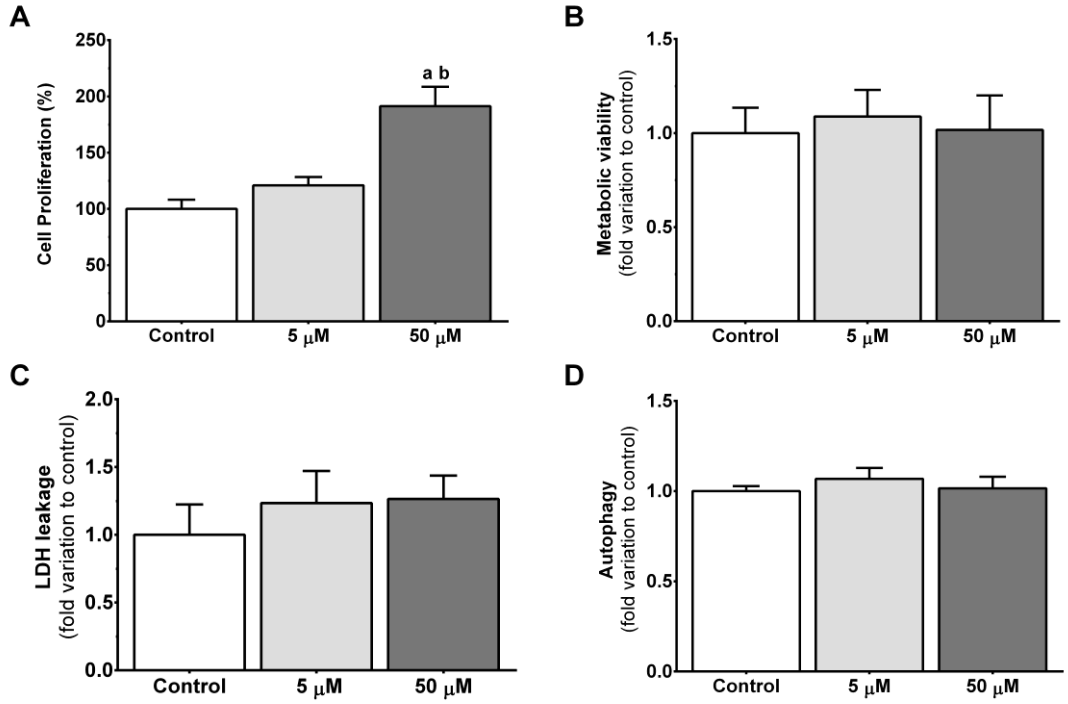


Figure 3.13 Effect of L-theanine (5 and 50 µM) in human Sertoli cells (hSCs) survival. The figure shows pooled data of independent experiments, indicating hSCs proliferation (Panel A), metabolic viability (Panel B), LDH cytotoxicity (Panel C) and Autophagy (Panel D). Cell proliferation data are presented as percentage, where control value was set at 100%, while viability, LDH cytotoxicity and autophagy are presented as fold variation to the control. All results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to control; b – relative to 5 µM L-theanine.

Glucose consumption by hSCs was stimulated by exposure to the pharmacological dose of L-theanine

Non-exposed hSCs presented a glucose consumption of 7.23 ± 3.85 pmol/cell. The addition of 5 µM of L-theanine to hSCs culture medium did not affect glucose consumption (8.74 ± 2.11

pmol/cell) in comparison to the control group. On the other hand, there was an increase (to 28.55 ± 7.24 pmol/cell) in glucose consumption of hSCs exposed to the pharmacological dose of L-theanine (50 µM), relative not only to the control group, but also to the group of hSCs exposed to 5 µM of L-theanine (Figure 3.14A).

Lactate production was maintained after exposure to L-theanine

LDH protein levels were similar in hSCs from the control group and those cultured with 5 or 50 µM of L-theanine (1.07 ± 0.15 and 0.92 ± 0.11-fold variation to control, respectively) (Figure 3.14B). Besides, LDH activity of hSCs exposed to 5 or 50 µM of L-theanine did not change when compared to the control group (Figure 3.14C). Likewise, lactate production by hSCs was not altered by exposure to the selected doses of L-theanine (Figure 3.14D). In fact, non-exposed hSCs produced 12.75 ± 2.33 pmol/cell of lactate and hSCs exposed to 5 or 50 µM of L-theanine produced a similar amount (15.38 ± 1.51 and 12.91 ± 2.20 pmol/cell, respectively). Moreover, the protein levels of MCT4 in hSCs exposed to 5 or 50 µM of L-theanine were also like those in the control group (1.09 ± 0.30 and 1.08 ± 0.37-fold variation to control, respectively). In addition, alanine production by hSCs was not altered by exposure to L-theanine (data not shown).

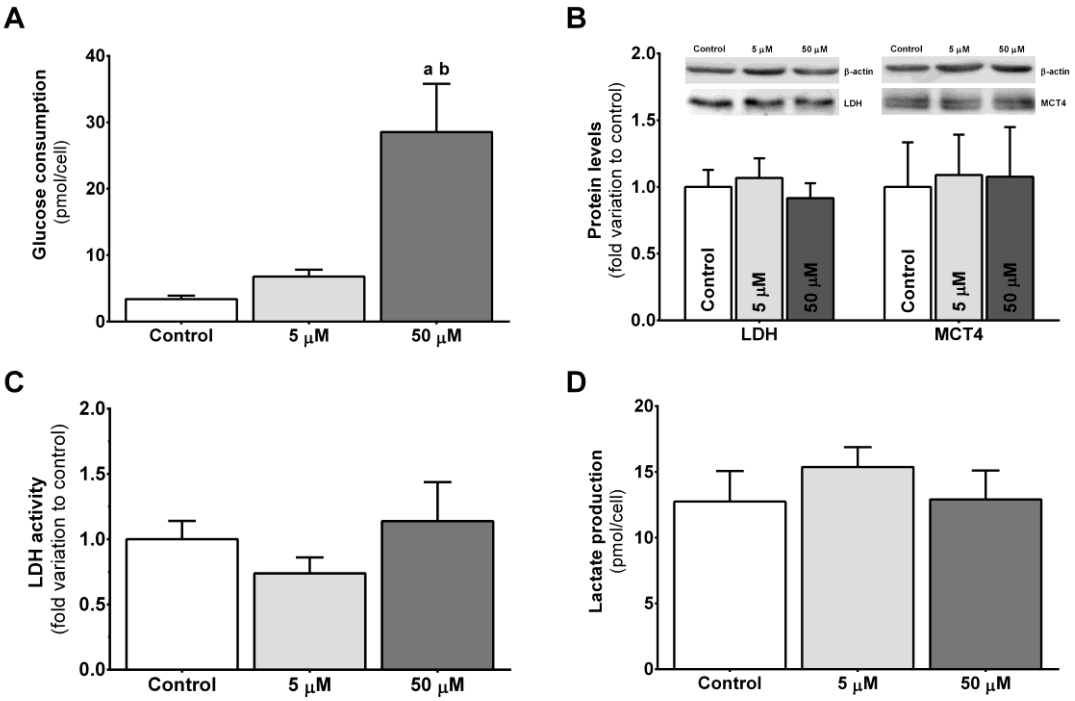


Figure 3.14 Effect of L-theanine (5 and 50 µM) in glucose metabolism of human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating glucose consumption (Panel A), LDH and MCT4 protein levels (Panel B), LDH activity (Panel C) and lactate production (Panel D). Panel B also displays the representative blots (of one sample) of LDH and MCT4. While glucose consumption and lactate production are presented as pmol/cell, results from protein levels and LDH activity are presented as fold variation to the control. All results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to control; b – relative to 5 µM L-theanine.

Mitochondrial membrane potential increased in hSCs exposed to the pharmacological dose of L-theanine

The addition of 5 or 50 µM of L-theanine to hSCs culture medium did not induce alterations in the protein levels of mitochondrial complexes I–V (Table 3.6). Additionally, the established mitochondrial membrane potential of hSCs was evaluated by the JC1 ratio and our results showed an increase in hSCs cultured with 50 µM of L-theanine (2.02 ± 0.25-fold variation to the control) relative to the control group. No differences were found among hSCs from the control group and those exposed to 5 µM of L-theanine (1.60 ± 0.24-fold variation to the control) (Figure 3.15A). Nevertheless, oxygen consumption was similar among the hSCs from the several experimental groups (Figure 3.15B).

Table 3.6 Protein expression levels of mitochondrial complexes in human Sertoli cells from the control group and groups exposed to 5 or 50 µM of L-theanine.

Mitochondrial complexes	Control	5 µM of L-theanine	50 µM of L-theanine
CI	1.00 ± 0.18	1.07 ± 0.06	0.98 ± 0.15
CII	1.00 ± 0.14	1.13 ± 0.17	0.90 ± 0.12
CIII	1.00 ± 0.09	0.99 ± 0.14	0.85 ± 0.06
CIV	1.00 ± 0.11	1.02 ± 0.11	0.86 ± 0.09
CV	1.00 ± 0.08	1.20 ± 0.22	0.89 ± 0.08

Abbreviations: CI, NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 8 (NDUFB8); CII, succinate dehydrogenase complex, subunit B, iron sulfur (SDHB); CIII, ubiquinol-cytochrome c reductase core protein II (UQCRC2); CIV, mitochondrially encoded cytochrome c oxidase I (MTCO1); CV, ATP synthase alpha-subunit (ATP5A). Results are expressed as mean ± SEM (fold variation to the control), n = 6 for each condition.

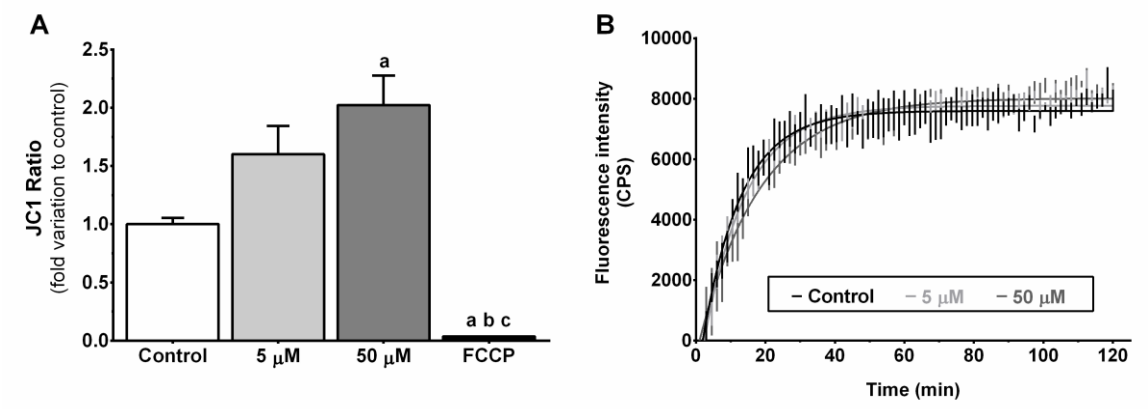


Figure 3.15 Effect of L-theanine (5 and 50 µM) in mitochondrial function of human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating mitochondrial membrane potential (JC1 ratio) (Panel A) and extracellular O_2 consumption (Panel B). Results are presented as fold variation to control. Results are expressed as mean ± SEM (n = 5 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to control; b – relative to 5 µM L-theanine; c – relative to 50 µM L-theanine. FCCP: Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone.

Exposure to L-theanine did not induce oxidative damage in hSCs

We have measured the levels of the end-products resultant from protein oxidation or nitration, as well as lipid peroxidation on hSCs after exposure to 5 or 50 μM of L-theanine. The addition of 5 or 50 μM of L-theanine to the culture medium of hSCs did not alter the levels of carbonyl groups, nitration or lipid peroxidation comparatively to the control group (Table 3.7).

Table 3.7 Oxidative damage levels, evaluated by carbonyl groups, nitration and lipid peroxidation, of human Sertoli cells from the control group and groups exposed to 5 or 50 μM of L-theanine.

Oxidative damage levels	Control	5 μM of L-theanine	50 μM of L-theanine
Carbonyl groups	1.00 ± 0.11	1.01 ± 0.12	1.05 ± 0.09
Nitration	1.00 ± 0.13	1.02 ± 0.14	1.05 ± 0.18
Lipid peroxidation	1.00 ± 0.11	0.92 ± 0.06	0.98 ± 0.14

Results are expressed as mean ± SEM (fold variation to the control), n = 6 for each condition.

Discussion

As SCs function is essential for the support of spermatogenesis and male fertility, in the present study, we used an *in vitro* model of hSCs to unveil the effects of L-theanine in these somatic testicular cells. L-theanine is considered safe for humans as no toxic effects were reported so far. Though, the regulation for its ingestion vary among countries. While in Japan L-theanine use in dietary products has no dose restrictions, in the USA, the Food and Drug Administration (FDA) considers it to be generally recognized as safe (GRAS), but recommends a maximum daily consumption of 1200 mg. Nevertheless, as its effects on cultured hSCs are not known, we evaluated the effect of two doses of theanine (5 μM - representing a quantity easily obtained through diet; and 50 μM - representing a pharmacological dose) in hSCs. This query was addressed by evaluating hSCs proliferation, metabolic viability, autophagy and LDH release after 24 h of exposure. The incubation with the lowest dose of L-theanine (5 μM) did not prompt any alterations in the considered parameters, while exposure of hSCs to the pharmacological dose of L-theanine (50 μM) induced an increase in cells proliferation when compared to the control group. These results suggest that the selected doses are not toxic for hSCs. Besides, as each Sertoli cell can only support a limited number of germ cells [8], a higher proliferation may be important to increase spermatozoa production and improve male reproductive potential. However, we must consider that in vivo the proliferation of adult SCs is limited, so data extrapolation should be made carefully. Still, this *in vitro* model of proliferating hSCs is largely established for toxicological studies. A recent study also reported the pro-proliferative action of L-theanine exposure (1–100 μM for 12 days) in cultured murine neuronal progenitor cells [9], thus corroborating our observations.

Generally, high proliferating cells require an increased uptake of nutrients. As hSCs can reprogram their metabolism to meet their needs, we evaluated the production/consumption of key metabolic metabolites. One of the major roles of hSCs is to metabolize glucose to produce pyruvate and lactate, so these substrates can be used for energy production by developing germ

cells. Hence, hSCs metabolism highly relies on glycolysis. Our results showed that the addition of a pharmacological dose of L-theanine (50 μM) to hSCs culture medium triggers a higher consumption of glucose, which is the main substrate available in the culture media (18 mM). A higher glucose uptake suggests that more pyruvate is being produced through glycolysis. Pyruvate is an important regulatory point of cells metabolism as it can follow three different pathways: 1) it can be converted to lactate by LDH; 2) it can produce alanine or 3) it can enter the mitochondria to form acetyl-CoA and fuel the Krebs cycle [1]. Both LDH protein expression levels and activity were similar between the experimental groups, resulting in a normal production of lactate in hSCs exposed to L-theanine. Moreover, the protein expression of MCT4, a specific monocarboxylate transporter that exports lactate to the extracellular medium, was also normal, showing that lactate production and export is not altered in the presence of L-theanine. Likewise, alanine production was similar between the experimental groups (data not shown). This led us to speculate that the higher pyruvate production resulting from the higher glucose uptake is being used by the mitochondria to produce metabolic intermediates necessary for anabolic processes that support the higher cellular proliferation. The fact that we did not detect acetate or citrate in the extracellular media (by the 1H-NMR analysis) supports that these metabolites are being used to fuel the Krebs cycle. In fact, an increased mitochondrial membrane potential was observed in the group of hSCs exposed to the pharmacological dose of L-theanine (50 μM), suggesting that L-theanine is influencing hSCs mitochondrial function. NADH reduced coenzyme produced from glycolysis contains electrons that have a high transfer potential. These electrons are removed from NADH and passed to oxygen by the electron transport chain (ETC – mitochondrial complexes I-IV) in the mitochondrial inner membrane. So, the increased glycolytic rates of hSCs exposed to the pharmacological dose of L-theanine (50 μM) induced an increased mitochondrial membrane potential. However, the protein expression of mitochondrial complexes (OXPHOS) was not altered, neither oxygen consumption. Still, in response to the detected metabolic changes, mitochondrial alterations could lead to an imbalanced ROS production and affect cellular proteins and lipids. However, our results show that hSCs oxidative profile was not altered by exposure to L-theanine. This may be due to a normal mitochondrial function or the antioxidant properties attributed to this amino acid. In fact, previous studies demonstrated that L-theanine is able to increase cellular antioxidant capacity due to its structural similarity to glutamic acid, as it is also a precursor of the main endogenous antioxidant glutathione [10].

Besides glycolysis, glutaminolysis is also a very important process to maintain the high metabolic rates of hSCs. Glutamine can be incorporated by hSCs through glutamate receptors, being hydrolyzed to glutamate by the enzyme glutaminase (EC 3.5.1.2). Consequently, glutamate may be excreted, or it can be further metabolized to yield α-ketoglutarate, a reaction-specific substrate for the Krebs cycle. L-theanine structure is very similar to that of glutamine, thus being able to bind to glutamate receptors in cells, although with lower affinity (80-fold difference). It has been reported that in brain cells, L-theanine may exert an agonist

or antagonist action on glutamate receptors in a dose and receptor-dependent manner [11]. Glutamine is a core metabolite for tumor cells proliferation. So, the inhibition of glutamate receptors by L-theanine is under investigation in many cancer studies, to find new ways to prevent tumor cells proliferation. In the present study, if an inhibition of glutamine uptake by hSCs was occurring, we would be able to measure these metabolites in the extracellular media by the 1H-NMR. As this was not the case and it was initially present in the extracellular media (3 mM), it was consumed by the hSCs. Moreover, as L-theanine is a precursor of glutamate, at the pharmacological dose it seems to be an extra source of glutamate for hSCs, thus explaining why this group of cells is proliferating more. Previous in vitro studies with other cells suggested that even if L-theanine cannot be metabolized through glutaminolysis, it is similarly incorporated into cells cytoplasm and can exert a glutamate-like effect [9]. A stimulation of glycolysis by glutamate was previously reported [12], as demonstrated in our study.

Our data indicate that exposure of hSCs to a pharmacological dose of L-theanine (50 μM) prompts an increase in cells proliferation and a higher glucose metabolization. This leads to an increased glycolytic rate to maintain the pools of Krebs cycle intermediates for ATP production and cellular components synthesis, to support the anabolic processes needed for building new cells. Although the mechanisms by which these metabolic alterations induce cell growth and proliferation remain poorly understood, the proliferative rates are correlated with substrate availability and cellular metabolism. Besides, a higher proliferation of hSCs may be important to maintain spermatogenesis and improve male reproductive potential. The glutamine-like effects of L-theanine reinforce the complementary action between glucose and glutaminolysis in hSCs metabolic function. Overall, our results support that a pharmaceutical L-theanine supplementation may be used to prevent or counteract spermatogenesis disruptions caused by some health conditions.

References

1. Dias TR, et al. (2013) Glucose Transport and Metabolism in Sertoli Cell: Relevance for Male Fertility. Current Chemical Biology 7(3):282-93.
2. Dias TR, et al. (2015) Dose-dependent effects of caffeine in human Sertoli cells metabolism and oxidative profile: Relevance for male fertility. Toxicology 328:12-20.
3. Dias TR, et al. (2017) Implications of epigallocatechin-3-gallate in cultured human Sertoli cells glycolytic and oxidative profile. Toxicology in Vitro 41:214-22.
4. Dias TR, et al. (2016) The single and synergistic effects of the major tea components caffeine, epigallocatechin-3-gallate and l-theanine on rat sperm viability. Food and function 7(3):1301-05.
5. Dias TR, et al. (2016) White tea intake prevents prediabetes-induced metabolic dysfunctions in testis and epididymis preserving sperm quality. The Journal of Nutritional Biochemistry 37:83-93.
6. Steger K, et al. (1996) Immunohistochemical detection of immature Sertoli cell markers in testicular tissue of infertile adult men: a preliminary study. International journal of andrology 19(2):122-28.
7. Scheid L, et al. (2012) Kinetics of L-Theanine Uptake and Metabolism in Healthy Participants Are Comparable after Ingestion of L-Theanine via Capsules and Green Tea–4. The Journal of nutrition 142(12):2091-96.
8. Oliveira PF and Alves MG (2015) The Sertoli cell at a glance. Sertoli Cell Metabolism and Spermatogenesis, Springer, pp. 3-13
9. Takarada T, et al. (2016) Possible activation by the green tea amino acid theanine of mammalian target of rapamycin signaling in undifferentiated neural progenitor cells in vitro. Biochemistry and Biophysics Reports 5:89-95.

10. Vuong QV, et al. (2011) L-Theanine: properties, synthesis and isolation from tea. Journal of the Science of Food and Agriculture 91(11):1931-39.
11. Kakuda T, et al. (2000) Protective effect of γ-glutamylethylamide (theanine) on ischemic delayed neuronal death in gerbils. Neuroscience letters 289(3):189-92.
12. Loaiza A, et al. (2003) Glutamate triggers rapid glucose transport stimulation in astrocytes as evidenced by real-time confocal microscopy. Journal of Neuroscience 23(19):7337-42.

Chapter 4

II - Ex Vivo Studies

This chapter was adapted from the following publications:

a) **Dias TR**, Alves MG, Tomás GD, Socorro S, Silva BM, Oliveira PF. (2014) "White tea as a promising antioxidant media additive for sperm storage at room temperature: a comparative study with green tea". Journal of Agricultural and Food Chemistry 62(3): 608–617 (DOI:10.1021/jf4049462).

b) **Dias TR**, Alves MG, Casal S, Silva BM, Oliveira PF. (2016) "Single and synergistic effect of major tea components caffeine, epigallocatechin gallate and L-theanine in rat sperm viability". Food & Function 7(3):1301-1305 (DOI:10.1039/C5FO01611H).

White tea as a promising antioxidant media additive for sperm storage at room temperature: a comparative study with green tea

Abstract

Refrigerated sperm storage reduces cells viability, due to oxidative unbalance. Unfermented teas present high levels of catechin derivatives, known to reduce oxidative stress. Herein, we investigated white tea (WTEA) effect on epididymal spermatozoa survival at room temperature (RT), using green tea (GTEA) for comparative purposes. The chemical profiles of WTEA and GTEA aqueous extracts were evaluated by proton nuclear magnetic resonance (^{1}H-NMR). Epigallocatechin gallate (EGCG) was the most abundant catechin, being twice as abundant in WTEA extract. The antioxidant power of storage media was evaluated, and spermatozoa antioxidant potential, lipid peroxidation and viability were assessed. The media antioxidant potential was higher when supplemented with WTEA, which was concomitant with the highest increase in sperm antioxidant potential and the decrease in lipid peroxidation. WTEA supplementation maintained spermatozoa viability to values like the obtained at the collection time. These findings provide evidence that WTEA extract is an excellent media additive for RT sperm storage, to facilitate transport and avoid refrigeration deleterious effects.

Keywords: antioxidants; *Camellia sinensis;* epigallocatechin gallate; green tea; reactive oxygen species; sperm; white tea.

Introduction

Tea (*Camellia sinensis* (L.)) is one of the most consumed beverages in the world and its medicinal properties have been widely explored [1]. It can be classified in three types: unfermented (green and white tea), partially fermented (oolong tea) and completely fermented (black tea) [2]. To produce green tea (GTEA), freshly harvested leaves are steamed to inactivate polyphenol oxidase enzyme and then rolled and dried. Its chemical composition is very similar to the fresh tea leaf [1]. White tea (WTEA) is exclusively prepared from young tea leaves or buds, harvested before being fully opened. The tea materials are picked and immediately steamed and dried to prevent oxidation, frequently followed by polymerization [3]. Unfermented teas have high polyphenolic content, mainly catechin derivatives, being epigallocatechin gallate (EGCG) the most abundant and powerful antioxidant [4]. With respect to processing, there are very little differences between green and white teas, although several reports suggest that WTEA present higher levels of antioxidants than GTEA [5]. Recently, antioxidant components have aroused great interest due to their ability to minimize the deleterious effects of reactive oxygen species (ROS) on a number of biological and pathological processes [6]. ROS are necessary for the normal physiological function of sperm [7], although its concentration must be kept under strict control to avoid deleterious effects, such as

damages to lipids in cell membranes, proteins, and DNA [8]. ROS overproduction may result in oxidative stress (OS), which is related to male subfertility and infertility [9]. In fact, spermatozoa are particularly vulnerable to such stress because ROS readily attack the polyunsaturated fatty acids (PUFAs) of the cells' membrane, initiating a self-propagating chain reaction. End-products of these lipid peroxidation reactions, such as malondialdehyde (MDA), are especially dangerous for cells viability [10]. Therefore, there is a growing interest in enlightening the role of ROS formation in sperm as they may result in poor sperm quality in freshly collected semen, as well as after semen processing for assisted reproductive technology (ART), such as artificial insemination (AI), *in vitro* fertilization (IVF) or cryopreservation [11].

The maintenance of mammalian sperm at room temperature (RT) for short-term periods is advantageous as the storage of sperm in a refrigerated environment induces a rapid decline in cells viability [12]. Establishment of an optimal composition for sperm storage medium is of extreme relevance, as spermatozoa are highly dependent on the supply of exogenous substrates and, due to their high metabolic rates, produce elevated amounts of ROS [12]. The addition of GTEA polyphenols has proven to be of great significance on frozen-thawed spermatozoa motility [13]. Hereupon, we aimed to investigate the possible protective effect of WTEA extract on epididymal spermatozoa survival at RT, using GTEA for comparative purposes [14]. The chemical profile of a WTEA and GTEA aqueous extracts was determined by proton nuclear magnetic resonance (^{1}H-NMR), as well as the antioxidant potential of sperm storage media containing these extracts. Furthermore, the effect of both extracts on epididymal spermatozoa maintenance at RT during 24, 48 and 72 h was evaluated by determining the spermatozoa antioxidant potential, lipid peroxidation and viability during that timeframe.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specifically stated.

Tea extracts

WTEA and GTEA were purchased on the Portuguese market (Diese, Organic Agriculture). Samples (n = 5) were subjected to infusion (1 g/100 mL distilled water; pH 5.5) at 100°C during 3 min, according to the manufacturer's instructions. The resulting infusions were filtered with qualitative filter papers (Cat. No. 516-0819, VWR, Leuven, France) in a vacuum system and freeze-dried overnight in a ScanVac CoolSafe Freeze Dryer™ (Labogene, Lynge, Denmark). The mean extraction yield (g of lyophilized extract per 100 g of dried teas leaves) was 25% for WTEA, and 20% for GTEA. The lyophilized extracts were kept in a desiccator, protected from light, until analysis.

Proton nuclear magnetic resonance spectroscopy

^{1}H-NMR spectra were acquired as previously described [15]. Briefly, ^{1}H-NMR spectra of WTEA and GTEA aqueous extracts dissolved in deuterium oxide (D_2O) were acquired at 14.1 T, 25°C, using a Bruker Avance 600 MHz spectrometer equipped with a 5-mm QXI probe and a z-gradient. ^{1}H-NMR spectra were acquired with solvent-suppression and a sweep width of 6 kHz, using a delay of 14 s, a water pre-saturation of 3 s, a pulse angle of 45°, an acquisition time of 3.5 s and at least 128 scans. Sodium fumarate (in final concentration of 1 mM) was used as internal reference (6.50 ppm) [16, 17], to quantify the extract compounds whenever present in solution. The following coupling patterns, available in the literature [18-23], were used to quantify the identifiable extract compounds (multiplet, ppm): L-theanine (triplet, 1.08); lactate (doublet, 1.33); alanine (doublet, 1.45); EGCG (doublet, 2.7); caffeine (singlet, 3.29); H1-α-glucose (doublet, 5.22); sucrose (doublet, 5.4); epigallocatechin (EGC) (singlet, 6.6); epicatechin (EC) (singlet, 7.0) (representative image in Figure 4.1). The relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSproTM NMR spectral analysis program (Acorn, NMR Inc., Fremont, CA, USA).

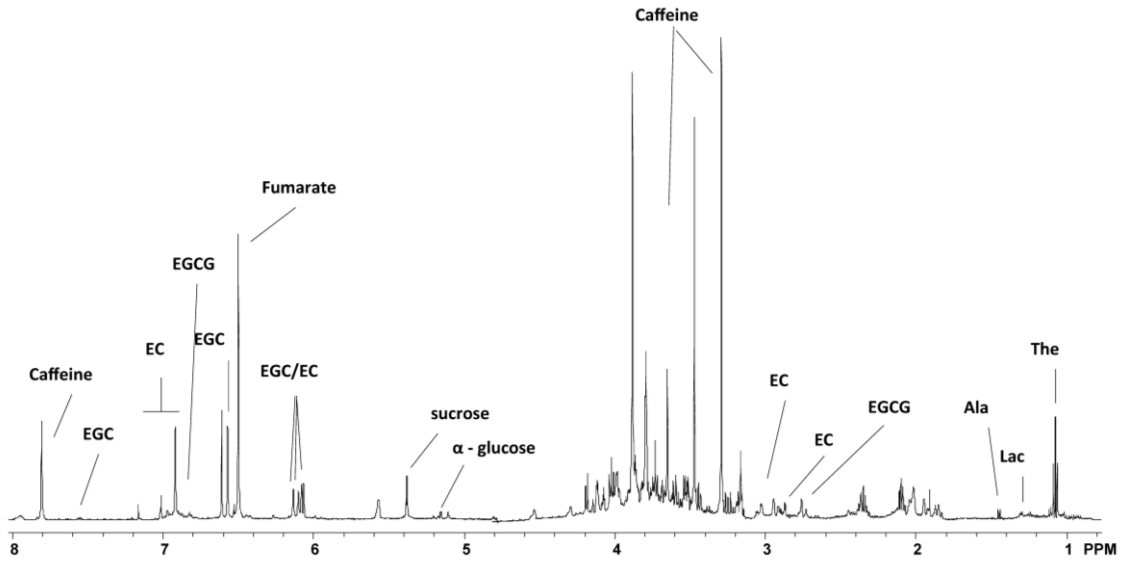


Figure 4.1 Representative proton nuclear magnetic resonance spectrum of white tea extract showing the phytocomponents peak assignments. EC, epicatechin; EGC, epigallocatechin; EGCG, epigallocatechin gallate; Ala, alanine; The, theanine; Lac, lactate.

Isolation of epididymal spermatozoa

The present study used six male Wistar rats 3-months old, obtained from our accredited animal colony (Health Sciences Research Centre, University of Beira Interior) and maintained on *ad libitum* food and water in a constant RT (20 ± 2°C) on a 12 h cycle of artificial lighting. Rats were fed with a standard chow diet (4RF21 certificate, Mucedola, Italy). All animal experiments were performed according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the

European directives for the care and handling of laboratory animals (Directive 86/609/EEC), after approbation by the National Ethics Committee of Animal Welfare.

Animals were anesthetized, by an intraperitoneal injection of a mixture of 90 mg/kg of ketamine and 10 mg/kg of xylazine and euthanized by cervical displacement. Cauda epididymis were isolated and immediately placed separately in a pre-warmed (37°C) Krebs-Ringer bicarbonate (TYH) medium (118.8 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.56 mM glucose, 1.01 mM sodium pyruvate, sodium 29.2 mM lactate, supplemented with 4.00 mg/mL bovine serum albumin (BSA), 0.06 mg/mL potassium penicillin G and 0.05 mg/mL streptomycin sulfate), prepared on the day of the experiment, as described by Toyoda [24]. Both epididymal cauda from each animal were minced together with a scalpel blade, to allow sperm to disperse into the medium, and the suspension was then incubated for 1.5 h at 37°C. The number of spermatozoa was determined using a hemocytometer and 8 x 10^5 spermatozoa were placed in 300 µL of a control medium (96.66 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$; 4.15 mM $NaHCO_3$, 5.56 mM D-Glucose, 0.33 mM sodium pyruvate, 23.28 mM sodium lactate, 20.85 hydroxyethyl piperazineethanesulfonic acid (HEPES), supplemented with 4.00 mg/mL BSA, 0.06 mg/mL potassium penicillin G, 0.05 mg/mL streptomycin sulfate) as previously described [25]. Moreover, the same number of spermatozoa was placed in four other media, with the same composition of the control group but containing additional concentrations of freeze-dried WTEA or GTEA aqueous extracts to a final concentration of 0.5 or 1 mg/mL. Subsequently, sperm suspensions were kept at acclimatized RT (22-23°C) during 24, 48 and 72 h. These sperm suspensions were used for the ferric reducing antioxidant power (FRAP), thiobarbituric acid reactive substances (TBARS) and sperm viability assays, after being processed as described in each section.

Protein quantification

After 72 h of incubation, spermatozoa were separated from the storage media through a centrifugation at 5000 x g for 15 min, 4°C. Then, sperm pellets were washed with 100 µL of phosphate-buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) and centrifuged again at 5000 x g for 15 min, 4°C. Total proteins were isolated from the spermatozoa by the addition of an adequate amount of PBS, followed by a sonication for 15 min at 4°C. Protein concentration was determined by BioRad (Hemel Hempstead, UK) Bradford micro-assay according to the manufacturer's instructions. Sperm suspensions were used in FRAP and TBARS assays.

Ferric Reducing Antioxidant Power assay

The FRAP of the media samples and spermatozoa pellets was performed according to the colorimetric method described by Benzie and Strain [26]. Briefly, working FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 2,4,6-Tripyridyl-s-Triazine (TPTZ) (10 mM

in 40 mM HCl) and $FeCl_3$ (20 mM) in a 10:1:1 ratio (v:v:v). A total of 180 µL of this reagent was mixed with 6 µL of each sample. The reduction of the Fe^{3+}-TPTZ complex to a colored Fe^{2+}-TPTZ complex by the samples was monitored immediately after adding the sample and 40 min later, by measuring the absorbance at 595 nm using an Anthos 2010 microplate reader (Biochrom, Berlin, Germany). Antioxidant potential of the samples was determined against standards of ascorbic acid, which were processed in the same manner as the samples. Absorbance results were corrected using a blank, with water instead of sample. The changes in absorbance values of test reaction mixtures were used to calculate the FRAP value as described elsewhere [26].

Thiobarbituric acid reactive substances assay

TBARS are formed as a byproduct of lipid peroxidation, which can be detected by the TBARS assay using thiobarbituric acid (TBA) as a reagent. This peroxidation reaction produces MDA, which reacts with TBA in conditions of high temperature and low pH, generating a pink colored complex that absorbs at 532 nm [27]. The TBARS assay was carried out by the method described by Iqbal and collaborators [28], with slight adaptations. Briefly, the reaction mixture in a total volume of 0.1 mL contained: 0.01 mL of the sample, 0.01 mL Tris-HCl buffer (150 mM, pH 7.1), 0.01 mL ferrous sulfate (1.0 mM), 0.01 mL ascorbic acid (1.5 mM) and 0.06 mL H_2O. This mixture was incubated at 37°C for 15 min. The reaction was stopped by addition of 0.1 mL of trichloroacetic acid (10% w/v). Subsequently, 0.2 mL of TBA (0.375% w/v) were added and all samples were incubated for 15 min at 100°C. Finally, samples were subjected to a centrifugation at 3000 x g for 10 min, 4°C. The amount of MDA formed in each of the samples was estimated by measuring optical density at 532 nm using a UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan) against a blank. The results were expressed as nmol of TBARS/mg protein.

Sperm viability evaluation

To assess sperm viability an eosin/nigrosin staining was used because it is effective, simple and, in addition of allowing sperm to be readily visualized, permits to assess sperm membrane integrity. This method was performed with slight modifications of a method previously described [29]. A drop of 5 µL of the sperm suspensions was mixed with 10 µL of 0.5% eosin/nigrosin stain and placed on a pre-warmed glass microscope slide. Samples were analyzed at 0, 24, 48 and 72 h of the experiment. The number of viable and non-viable spermatozoa was determined counting a total of 333 spermatozoa per slide in continuous random fields under an optical microscope, with oil immersion (x1000 magnification), to determine the percentage of viable sperm, as previously described [16]. Live sperm remained white, while dead sperm stained pink, because the integrity of their plasma membrane was compromised leading to the uptake of the dye (Figure 4.2).

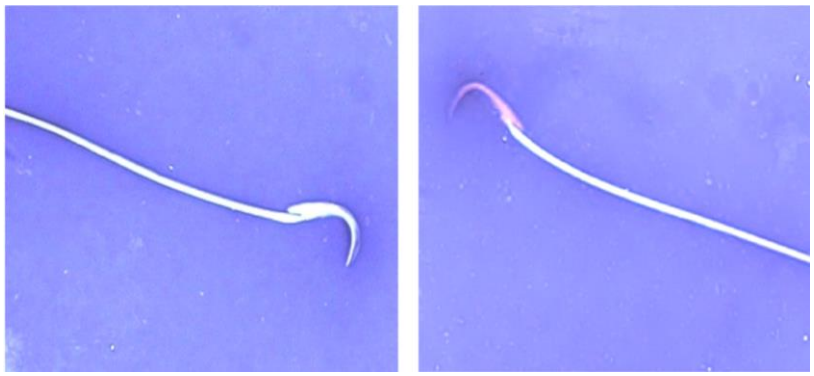


Figure 4.2 Microscopic image of rat spermatozoa after eosin/nigrosin staining: viable spermatozoon (left) and non-viable spermatozoon (right).

Statistical analysis

Statistical significance was assessed by two-way ANOVA, followed by Bonferroni post-test using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). All data are presented as mean ± SEM. Differences with $p < 0.05$ were considered statistically significant. Further analysis of the statistical power (SP) of differences of experimental data was evaluated with a one-tail test assuming an alpha of 0.05 that corresponds to a 0.95 confidence interval, as described by Levin [30], using the online software: http://www.dssresearch.com/KnowledgeCenter/toolkitcalculators/statisticalpowercalculators.aspx.

Results

White tea extract presents the highest content in catechin derivatives

Tea has been characterized by its high content in flavonoids [31], such as catechin derivatives and other polyphenols [32], which have biological and pharmaceutical properties that have been linked to beneficial effects on human health [33]. The ^{1}H-NMR data obtained from the aqueous extracts allowed the assignment of following phytochemicals: three catechin derivatives - EC, EGC and EGCG; one methylxanthine – caffeine; two free amino acids - L-theanine and alanine; two carbohydrates – glucose and sucrose; and one organic acid – lactate. As expected, the most abundant class of phytochemicals was the catechins family representing 133 ± 14 g/kg and 91 ± 7 g/kg of WTEA and GTEA extracts, respectively. Among catechins, EGCG was the most abundant in both tea extracts, though in WTEA extract the quantity of this compound (82 ± 7 g/kg of WTEA extract) was nearly twice the quantified in GTEA extract (42 ± 2 g/kg of GTEA extract). Concerning caffeine and sucrose, WTEA extract also demonstrated a higher amount (71 ± 8 and 60 ± 4 g/kg of WTEA extract, respectively) when compared with GTEA (21 ± 3 and 26 ± 2 g/kg of GTEA extract, correspondingly). L-theanine, alanine and glucose were also present in considerable amounts accounting for 19 ± 2, 0.7 ± 0.1 and 6 ± 1 g/kg of WTEA extract and 22 ± 1, 0.20 ± 0.04 and 11 ± 2 g/kg of GTEA extract, respectively (Table 4.1).

Epicatechin gallate (ECG), catechin (C) and gallocatechin gallate (GCG) were absent in our extracts. Generally, these polyphenols are minor tea components but sometimes are absent in tea extracts. For instance, Carvalho and collaborators [34] and Rusak and collaborators [3] also did not found GCG and C in their tea extracts, respectively. Hence, the white and green teas qualitative phytochemical profiles obtained using ^{1}H-NMR (Table 4.1) are in accordance to the phenolic and methylxanthine profiles obtained by HPLC/UV, HPLC/DAD or HPLC/MS previously reported by other authors [3, 34, 35]. The observed differences in the quantitative profile may be mainly due to the use of different extraction conditions (solvents, temperatures, extraction time and leaves/water ratio). Also, the natural variability of plants caused by edapho-climatic factors, harvesting techniques or agricultural practices may contribute to these differences.

Table 4.1 Phytochemical profile of white tea (WTEA) and green tea (GTEA) extracts determined by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy (g of compound/kg of tea extract).

Compound	Content (g of compound/kg of tea extract)	
	WTEA	**GTEA**
Glucose	6 ± 1.00	11 ± 2.00
Sucrose	60 ± 4.00	26 ± 2.00
Lactate	0.40 ± 0.01	0.46 ± 0.02
Alanine	0.7 ± 0.10	0.20 ± 0.04
Caffeine	71 ± 8.00	21 ± 3.00
L-Theanine	19 ± 2.00	22 ± 1.00
EC	5 ± 1.00	28 ± 2.00
EGC	46 ± 6.00	21 ± 3.00
EGCG	82 ± 7.00	42 ± 2.00

Abbreviations: EC, epicatechin; EGC, epigallocatechin; EGCG, epigallocatechin gallate. Results are presented as mean ± SEM (n = 5).

Storage media supplemented with white tea extract have the highest antioxidant potential

As the aqueous extracts of WTEA and GTEA demonstrated to be rich in catechin derivatives, namely in EGCG, we performed a FRAP assay to evaluate the antioxidant potential of all the experimental media supplemented with these extracts in comparison to the control medium. The FRAP assay measures the potential of an antioxidant to reduce ferric (III) to ferrous (II) in a redox-linked colorimetric reaction that involves single electron transfer [36]. The reducing power of a compound/extract serves as an indicator of its potential antioxidant activity (FRAP value). The control medium showed a FRAP value of 1.64 ± 0.05 µmol of antioxidant potential/L (Figure 4.3). On the other hand, the FRAP value of the media supplemented with WTEA and GTEA revealed a dose-dependent reducing power (Figure 4.3), with higher FRAP values relatively to the control group during all the experiments (SP = 100%). Comparing GTEA supplementation, the medium with 1 mg/mL of GTEA extract presented a higher antioxidant potential (11 ± 0.2 µmol of antioxidant potential/L) comparatively to the medium with 0.5 mg

of GTEA extract/mL (6 ± 0.3 µmol of antioxidant potential/L) (SP = 100%). In relation to WTEA, the FRAP value of the medium supplemented with 0.5 mg/mL of WTEA extract (14 ± 0.3 µmol of antioxidant potential/L) was higher comparatively to the medium with the same concentration of GTEA extract (SP = 100%). Moreover, the medium containing 1 mg/mL of WTEA presented a higher antioxidant potential (24 ± 0.6 µmol of antioxidant potential/L) relative to the media supplemented with 0.5 mg/mL of WTEA extract and 1 mg/mL of GTEA extract (SP = 100%).

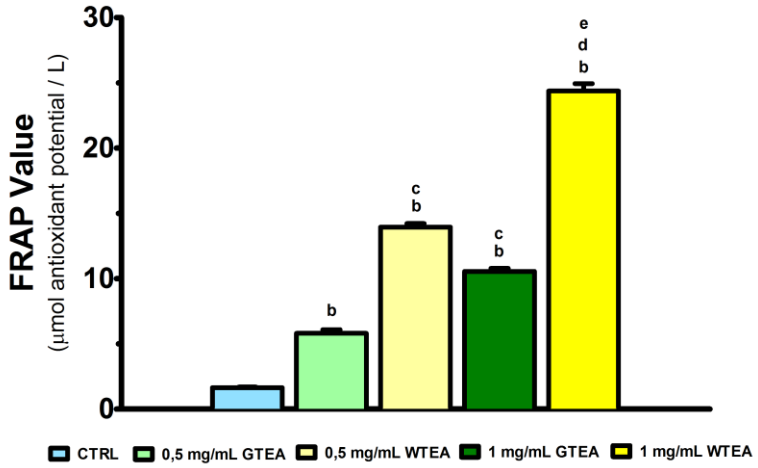


Figure 4.3 Ferric reducing antioxidant power (FRAP) of the epididymal spermatozoa storage media, control medium (CTRL) and media supplemented with freeze-dried white tea (WTEA) or green tea (GTEA) aqueous extracts to a final concentration of 0.5 or 1 mg/mL. The antioxidant power is expressed by the FRAP value (µmol of antioxidant potential/L). Results are presented as mean ± SEM (n = 6). Significantly different results ($p < 0.05$) are indicated as: b – relative to CTRL; c – relative to 0.5 mg of GTEA extract/mL; d – relative to 0.5 mg of WTEA extract/mL; e – relative to 1 mg of GTEA extract/mL.

White tea extract increased the antioxidant potential of spermatozoa

As the media supplemented with WTEA extract showed the highest antioxidant potential, we expected to verify the same profile in the antioxidant potential of the spermatozoa. Therefore, we also measured the antioxidant potential of the spermatozoa pellets at 24, 48 and 72 h with the FRAP assay. Accordingly, spermatozoa incubated in control medium had a lower antioxidant potential than spermatozoa stored in the media supplemented with WTEA or GTEA extracts after 48 and 72 h of storage (Figure 4.4). At 48 h, spermatozoa stored in the medium with 0.5 mg of WTEA extract/mL showed a higher FRAP value (2.8 ± 0.2 µmol of antioxidant potential/µg of protein) relative to spermatozoa from the control medium (1.1 ± 0.3 µmol of antioxidant potential/µg of protein) and those culture with 0.5 mg of GTEA extract/mL (1.4 ± 0.1 µmol of antioxidant potential/µg of protein) (SP =100%). At 72 h, the FRAP value of spermatozoa incubated with 0.5 and 1 mg of GTEA extract/mL (1.5 ± 0.2 (SP = 84%) and 1.8 ± 0.1 (SP = 100%) µmol of antioxidant potential/µg of protein, respectively) was higher relative to the control group. Besides, at 72h, the FRAP value determined for spermatozoa kept in the control medium was lower than at 48 h, reaching a value of 0.1 ± 0.3 µmol of antioxidant potential/µg of protein (SP = 100%) (Figure 4.4). Contrastingly, the antioxidant potential of spermatozoa stored in the media supplemented with WTEA extract

(0.5 and 1.0 mg of WTEA extract/mL) was remarkably higher than that observed at 48 h (8.4 ± 2.7 (SP = 99.9%) and 12.2 ± 3.1 (SP = 100%) µmol of antioxidant potential/µg of protein, respectively) and relative to the control group. Moreover, at this timepoint, there were no differences between both concentrations of the same extract.

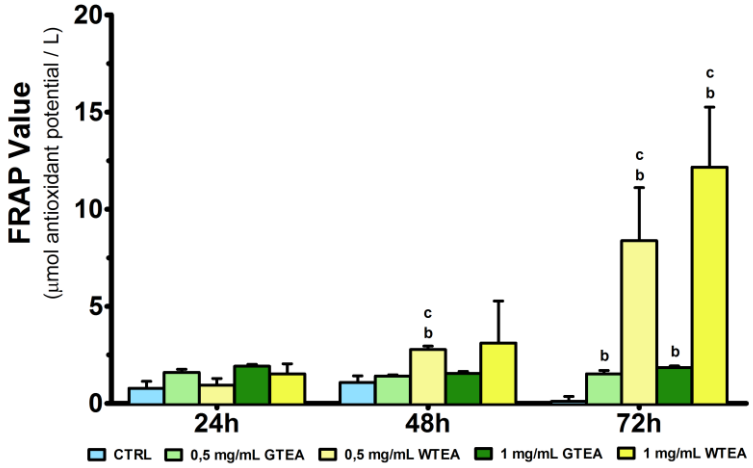


Figure 4.4 Ferric reducing antioxidant power (FRAP) of the epididymal spermatozoa stored in control medium (CTRL) and media supplemented with freeze-dried white tea (WTEA) or green tea (GTEA) aqueous extracts to a final concentration of 0.5 or 1 mg/mL, during the 24, 48 and 72 h of the experiment. The antioxidant power is expressed by the FRAP value in µmol of antioxidant potential/µg of protein and is presented as mean ± SEM (n = 6). Significantly different results ($p < 0.05$) are indicated as: b – relative to CTRL; c – relative to 0.5 mg of GTEA extract/mL.

Spermatozoa lipid peroxidation was reduced by white tea supplementation

During spermatozoa storage, ROS overproduction may induce several deleterious effects [37] such as lipid peroxidation. As spermatozoa antioxidant potential increased after incubation with the tea extracts, we hypothesized that it could also control lipid peroxidation. Therefore, we performed the TBARS assay to determine the levels of lipid peroxidation that occurred in spermatozoa during storage in the different media. ROS have extremely short half-lives; thus, they are difficult to measure directly. Instead, several products induced by OS, such as TBARS, can be measured and used as an accurate indicator of OS [38]. We observed a decrease on lipid peroxidation in spermatozoa stored in media supplemented with WTEA and GTEA extracts. At 24 h, there was a decrease in lipid peroxidation in spermatozoa stored in the media containing 0.5 mg/mL of GTEA (47 ± 7 nmol TBARS/mg protein) and 0.5 or 1 mg/mL of WTEA (54.8 ± 4.7 and 46 ± 2 nmol TBARS/mg protein, respectively) relative to spermatozoa in the control medium (73 ± 4 nmol TBARS/mg protein) (SP = 100%) (Figure 4.5). After 48 h, lipid peroxidation decreased from 81 ± 10 nmol TBARS/mg protein in the control group to 44 ± 6 and 41 ± 8 nmol TBARS/mg protein (SP = 100%) in spermatozoa stored in the media supplemented with 0.5 or 1 mg of WTEA extract/mL, respectively (Figure 4.5). Moreover, spermatozoa stored in the medium with 1 mg of WTEA extract/mL also presented less lipid peroxidation (41 ± 8 nmol TBARS/mg protein) than the spermatozoa maintained in the medium with 1 mg of GTEA extract/mL (69 ± 10 nmol TBARS/mg protein) (SP = 99.9%). At the end of the experiment (72 h), the control group presented a lipid peroxidation of 79 ± 4 nmol TBARS/mg protein, which

decreased to 58 ± 8, 42 ± 5 and 41 ± 3 nmol TBARS/mg protein (SP = 100%) when supplemented with 0.5 mg of GTEA extract/mL or 0.5 and 1 mg of WTEA extract/mL, respectively. Additionally, lipid peroxidation in spermatozoa kept in the media with 0.5 and 1 mg of WTEA extract/mL was lower than in spermatozoa stored in the media supplemented with 0.5 (SP = 99.5%) and 1 mg of GTEA extract/mL (SP = 100%), correspondingly.

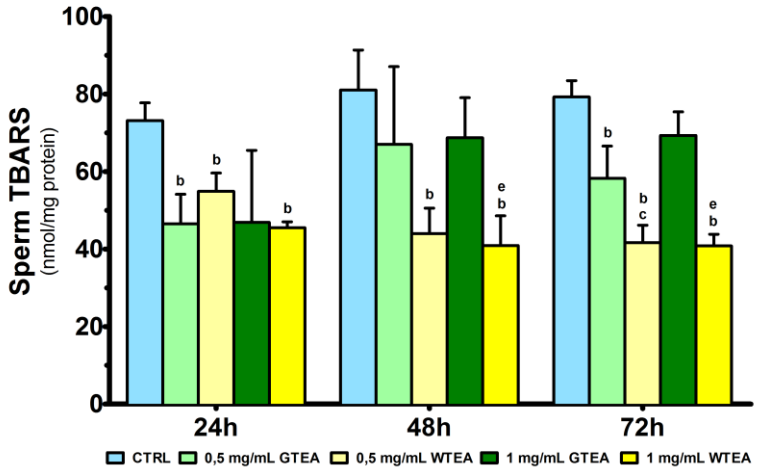


Figure 4.5 Sperm thiobarbituric acid reactive substances (TBARS) produced in epididymal spermatozoa stored in control medium (CTRL) and media supplemented with freeze-dried white tea (WTEA) or green tea (GTEA) aqueous extracts to a final concentration of 0.5 or 1 mg/mL, during the 24, 48 and 72 h of the experiment. Results are expressed in nmol/mg of protein and are presented as mean ± SEM (n = 6). Significantly different results ($p < 0.05$) are indicated as: b – relative to CTRL; c – relative to 0.5 mg of GTEA extract/mL; e – relative to 1 mg of GTEA extract/mL.

White tea increased sperm viability during room temperature storage

Sperm viability is one of the most important parameters to assess sperm quality [39]. As WTEA and GTEA supplementation increased the antioxidant potential of storage media and spermatozoa, while decreasing spermatozoa lipid peroxidation, we hypothesized that sperm viability could be improved by these extracts. Therefore, we evaluated the sperm viability at the time of spermatozoa collection (0 h) and at 24, 48 and 72 h of incubation. At collection, sperm viability averaged 67 ± 2%. During spermatozoa storage at RT in control medium we observed that viability was continuously decreasing, presenting values of 31 ± 2%, 19 ± 2% and 14 ± 1% at 24, 48 and 72 h, respectively (SP = 100%) (Figure 4.6). Noteworthy, there was a dose-dependent increase in viability, during the 3-day storage, in spermatozoa stored in media supplemented with both tea extracts when compared with the control. At 24 h, viability of spermatozoa kept in the media supplemented with any of the tea extracts was very similar to the observed at 0 h (Figure 4.6). At 48 h, viability of spermatozoa kept in the media with 0.5 mg/mL of GTEA or WTEA decreased (49 ± 2 and 53 ± 5%, respectively) relative to the collection time (SP = 100%). Conversely, viability corresponding to the spermatozoa stored in the media with 1 mg/mL of GTEA (59 ± 3%; SP = 100%) or WTEA (65 ± 3%; SP = 99.5%) was higher than the viability verified in the media containing half concentration of these extracts. The same profile was verified at 72 h of storage, with the only exception that spermatozoa

kept in 1 mg of WTEA extract /mL medium exhibited higher viability (63 ± 3%) in relation to the spermatozoa kept in the medium with 1 mg of GTEA extract/mL (49 ± 2%; SP = 100%). Remarkably, spermatozoa incubated in the medium with 1 mg of WTEA extract/mL averaged a viability of 65% throughout the 3-day storage at RT, matching the viability observed at 0 h (Figure 4.6).

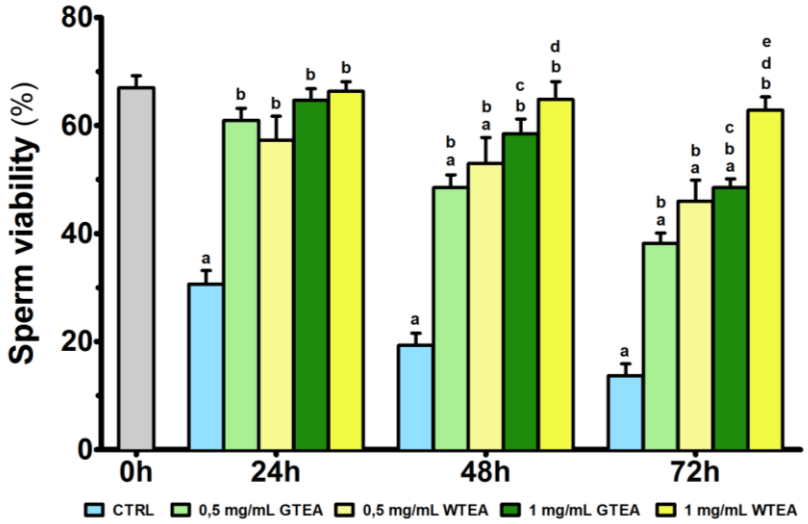


Figure 4.6 Spermatozoa viability at collection time (0 h) and during the 3-day storage in control medium (CTRL) and media supplemented with freeze-dried white tea (WTEA) or green tea (GTEA) aqueous extracts to a final concentration of 0.5 or 1 mg/mL. Results are presented as mean ± SEM (n = 6). Significantly different results ($p < 0.05$) are indicated as: a – relative to 0h; b – relative to CTRL; c – relative to 0.5 mg of GTEA extract/mL; d – relative to 0.5 mg of WTEA extract/mL; e – relative to 1 mg of GTEA extract/mL.

Discussion

Despite the similarities between GTEA and WTEA, the number of studies to investigate the health benefits of WTEA is negligible compared to GTEA. Recently, WTEA aroused great interest among investigators due to its high content in tea polyphenols [40]. Thus, WTEA showed the highest antioxidant activity when compared to other types of tea [2, 5]. Our WTEA extract was richer in flavonoids than the GTEA extract from the same brand. EGCG is known as one of the most powerful antioxidants and the most pharmacologically active catechin derivative [41]. WTEA extract showed a high concentration of EGCG (82 ± 7 g/kg of WTEA extract) relative to the GTEA extract (42 ± 2 g/kg of GTEA extract). Many studies reported that EGCG has a positive impact in a variety of human diseases depending on its concentration [2, 42, 43]. EGCG has a protective action against several deleterious effects of diseases due to its ability to counteract OS [44].

Recently, it was reported that EGCG therapy protects against testicular ischemia-reperfusion injury through its antioxidant activity [45]. Importantly, the addition of EGCG at low concentrations to the extracellular media of human spermatozoa improved their motility and viability after incubation at 37°C for 30 min [46]. Our results showed that WTEA is very rich in EGCG, representing 62% of the total catechins content. The lower content in EGCG observed in

GTEA extract may be reflected in the antioxidant properties of the extract [40]. In addition, caffeine content was previously reported to be higher in WTEA [40] and our extract presented a remarkably high caffeine content (71 ± 8 g/kg of WTEA extract) [47]. Besides, our WTEA extract also presented higher caffeine content comparatively to our GTEA extract (21 ± 3 g/kg of GTEA extract). This phytochemical differences, as well as the variation found between our GTEA extract composition and the ones verified by other authors [34], could be due to the natural variability of the plants. This is related to the geographical origin, climate and/or agricultural practices, and to differences in the extraction procedures (solvents, temperatures, times of extraction and ratio leaves/water) and analytical techniques [48, 49]. Moreover, different leaves processing also influences the tea chemical composition [40]. Nevertheless, caffeine is described to stimulate lipolysis, interfere with glucose and fatty acid metabolism, thus having an important role in cellular metabolism [50]. Noteworthy, the WTEA extract was also richer in total sugars content, which are important substrates for cellular metabolism. Our ^{1}H-NMR results are in accordance to the phenolic and methylxanthine profiles previously reported using other techniques, including HPLC/UV, HPLC/DAD or HPLC/MS [3, 34, 35].

Spermatozoa present high metabolic rates that are in close association with elevated amounts of OS [51], which is considered one of factors responsible for poor semen quality [52]. OS results from an uncontrolled ROS production that when exceeds spermatozoa antioxidant capacity become harmful, inducing membrane lipid peroxidation, compromising spermatozoa survival and fertilizing potential [53]. Epididymal spermatozoa survival and maintenance is crucial for both, natural and assisted reproduction [54]. The mammalian epididymis creates a unique microenvironment that allows the transformation of immotile immature spermatozoa into mature competent cells. Besides, it stores the mature and viable spermatozoa in cauda epididymis until ejaculation.

Cryopreservation and refrigeration of spermatozoa have been highly debated, and it has been proposed that the maintenance of spermatozoa at RT for short-term periods can be an effective alternative to avoid the rapid decline of sperm viability after storage in a refrigerated environment [12, 55]. So, there is a growing interest in the establishment of an optimal medium composition for sperm storage at RT. Several media have been developed, but spermatozoa viability after storage in those media is still very low and far from the ideal [12, 56-59]. A study using mouse sperm evaluated the effect of RT-storage in various bicarbonate and phosphate-based media used for IVF or embryo culture, and reported that the low bicarbonate and HEPES containing medium would better preserve spermatozoa *in vitro* [12]. The addition of substrates, such as glucose, further enhanced sperm survival, although the authors concluded that further tests concerning the addition of preserving agents were still required [12, 59]. Studies using sperm from different mammalian origin preserved at RT in a saline-buffered storage medium also showed that supplementation with serum or egg yolk could greatly increase sperm survival and function [56-58]. At the same time, those authors concluded that there was still a crucial requirement in controlling the production of ROS, which are more likely to be generated in a

RT system. Indeed, some studies have been made to assess the effect of tea catechins on sperm viability and survival [13, 46]. Several concentrations of EGCG (1, 50 and 100 μM) were tested as a supplement of the storage media and although the authors obtained encouraging results in the sperm fertilization ability of frozen–thawed sperm, they did not verify an increase in sperm survival after storage [13]. Therefore, we hypothesized that WTEA extract could modulate some important sperm functions, such as antioxidant capacity and lipid peroxidation, improving sperm viability during RT storage.

Using the FRAP assay we verified that the media containing WTEA extract presented a higher antioxidant potential (FRAP value), in a dose-dependent manner, than the media with GTEA extract. This suggests that the antioxidant potential of sperm storage medium is greatly increased by the addition of WTEA extract. Concomitantly, the antioxidant potential of spermatozoa cultured in media containing WTEA extract was also higher. The antioxidant potential of spermatozoa incubated with WTEA increases over time, suggesting a gradual incorporation of the antioxidant compounds present in the extract into the medium. The same profile was verified in spermatozoa stored with GTEA extract but in a much smaller extent, evidencing that WTEA extract provides the most potent antioxidant potential.

One of the most important deleterious effects caused by OS is lipid peroxidation because mammalian spermatozoa are rich in PUFAs that are highly vulnerable to ROS attack [10]. In OS conditions, spermatozoa lipid membranes are oxidized and the end-product of these reactions, MDA, can be measured by the TBARS assay. Our results showed that during the 3-day sperm storage at RT, WTEA extract was the most effective in decreasing the lipid peroxidation in spermatozoa. This may be related to the higher concentration of polyphenols in the WTEA extract, as polyphenols have a protecting action against lipid peroxidation in cells. At 24 h of storage, there was a decrease in lipid peroxidation in spermatozoa incubated with 0.5 mg/mL GTEA and those incubated with 0.5 or 1 mg/mL of WTEA relative to the control group. After 48 and 72 h, lipid peroxidation of sperm stored in the media supplemented with WTEA extract kept constant and similar to the observed at 24 h, while in sperm stored in the media containing GTEA extract the lipid peroxidation increased over time. This reflects a stronger ability of WTEA to prevent sperm lipid peroxidation during RT-storage.

Male infertility affects about 50% of couples [60]. Noteworthy, in most male patients with subfertility or infertility, the condition is due to loss of sperm function rather than the number of spermatozoa [61]. Therefore, spermatozoa viability is an essential parameter to evaluate sperm quality and to evaluate male factor infertility. The supplementation of the storage media with tea extracts increased sperm viability in a dose-dependent manner. OS is known to play a crucial role in the loss of functional competence and when ROS production is elevated, dysfunctional spermatozoa are produced [62]. It has also been reported a negative correlation between ROS production and sperm movement, evidencing the importance of ROS control in spermatozoa function [63]. High ROS levels are detrimental to fertility potential in natural and

assisted conception [64] and sperm capacitation can be lost due to OS [65]. Therefore, the higher increase on sperm viability in the groups stored in WTEA relative to those stored with GTEA may be due to the higher polyphenolic content in WTEA extract and consequently, to its higher antioxidant potential. Moreover, as the viability improvement was more effective with the highest dose of WTEA extract (1 mg/mL), we suggest that the best protection attained with the highest dose of WTEA extract may be responsible for the overall better results observed with this concentration. Nevertheless, the concentrations of EGCG observed in our extracts correspond to about 90-180 μM for WTEA and 45-90 μM for GTEA. These EGCG concentrations are in the same order of magnitude of those used by Kaedei and collaborators [13], who did not verify an increase in the survival of spermatozoa stored in EGCG supplemented media. Our results showed that tea supplementation highly improved spermatozoa survival, supporting the idea that the combined effect of all the tea components is responsible for the positive effects observed in sperm viability.

In conclusion, the addition of WTEA aqueous extract to spermatozoa storage media can be a good, simple and inexpensive strategy for short-term storage at RT. Our results indicate that WTEA extract improves spermatozoa viability by increasing the spermatozoa and storage media antioxidant potential and decreasing spermatozoa lipid peroxidation. Moreover, the first hours of sperm preservation are crucial and marked by an increase in OS that can be counteracted by WTEA polyphenols. More studies will be needed to fully disclose the molecular mechanisms behind these results. Nevertheless, the addition of WTEA aqueous extract to the standard sperm storage medium can reduce or even eliminate the limitations of the *in vitro* spermatozoa storage in a refrigerated environment and enable the sperm transport for posterior use in ART.

References

1. Moderno P, et al. (2009) Recent patents on *Camellia sinensis*: source of health promoting compounds. Recent Patents on Food, Nutrition and Agriculture 1(3):182-92.
2. Dias TR, et al. (2013) White Tea (*Camellia Sinensis* (L.)): Antioxidant Properties and Beneficial Health Effects. International Journal of Food Science, Nutrition and Dietetics 2(2):19-26.
3. Rusak G, et al. (2008) Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and the solvent used. Food Chemistry 110(4):852-58.
4. Higdon JV and Frei B (2003) Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. Critical Reviews in Food Science and Nutrition 43(1):89–143.
5. Sharangi A (2009) Medicinal and therapeutic potentialities of tea (*Camellia sinensis* L.)-A review. Food Research International 42(5-6):529-35.
6. Fiorentino A, et al. (2008) Isolation and structure elucidation of antioxidant polyphenols from quince (Cydonia vulgaris) peels. Journal of Agricltural and Food Chemistry 56(8):2660-67.
7. Hampl R, et al. (2012) Impact of oxidative stress on male infertility. Ceska Gynekologie 77(3):241-45.
8. Valko M, et al. (2007) Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry and Cell Biology 39(1):44-84.
9. Aitken RJ, et al. (2010) Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. Human Reproduction 25(10):2415-26.
10. Mylonas C and Kouretas D (1999) Lipid peroxidation and tissue damage. In Vivo 13(3):295-309.
11. Guthrie H and Welch G (2012) Effects of reactive oxygen species on sperm function. Theriogenology 78(8):1700-08.
12. Sato M and Ishikawa A (2004) Room temperature storage of mouse epididymal spermatozoa: exploration of factors affecting sperm survival. Theriogenology 61(7):1455-69.

13. Kaedei Y, et al. (2012) Effects of (−)-Epigallocatechin Gallate on the Motility and Penetrability of Frozen–Thawed Boar Spermatozoa Incubated in the Fertilization Medium. Reproduction in Domestic Animals 47(6):880-86.
14. Costa RM, et al. (2009) Evaluation of free radical-scavenging and antihemolytic activities of quince (Cydonia oblonga) leaf: a comparative study with green tea (Camellia sinensis). Food and Chemical Toxicology 47(4):860-65.
15. Alves MG, et al. (2011) Substrate selection in hearts subjected to ischemia/reperfusion: role of cardioplegic solutions and gender. NMR in Biomedicine 24(9):1029-37.
16. Rato L, et al. (2013) High-energy diets may induce a pre-diabetic state altering testicular glycolytic metabolic profile and male reproductive parameters. Andrology 1(3):495–504.
17. Alves MG, et al. (2012) In vitro cultured human Sertoli cells secrete high amounts of acetate that is stimulated by 17beta-estradiol and suppressed by insulin deprivation. Biochimica et Biophysica Acta 1823(8):1389-94.
18. Govindaraju V, et al. (2000) Proton NMR chemical shifts and coupling constants for brain metabolites. NMR in Biomedicine 13(3):129-53.
19. Ohno A, et al. (2011) Characterization of tea cultivated at four different altitudes using 1H NMR analysis coupled with multivariate statistics. Journal of Agricultural and Food Chemistry 59(10):5181-87.
20. Uekusa Y, et al. (2007) Dynamic behavior of tea catechins interacting with lipid membranes as determined by NMR spectroscopy. Journal of Agricultural and Food Chemistry 55(24):9986-92.
21. Lee J-E, et al. (2010) Geographical and climatic dependencies of green tea (*Camellia sinensis*) metabolites: a 1H NMR-based metabolomics study. Journal of Agricultural and Food Chemistry 58(19):10582-89.
22. Peres I, et al. (2010) NMR structural analysis of epigallocatechin gallate loaded polysaccharide nanoparticles. Carbohydrate Polymers 82(3):861-66.
23. Lee J-E, et al. (2011) 1H NMR-based metabolomic characterization during green tea (*Camellia sinensis*) fermentation. Food Research International 44(2):597-604.
24. Toyoda Y (1971) Studies on fertilization of mouse eggs in vitro. I. In vitro fertilization of eggs by fresh epididymal sperm. The Japanese journal of animal reproduction 16(4):147-51.
25. Quinn P, et al. (1982) Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. Journal of Reproduction and Fertility 66(1):161-68.
26. Benzie IF and Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical Biochemistry 239(1):70-76.
27. Ohkawa H, et al. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 95(2):351-58.
28. Iqbal M, et al. (1996) Glutathione metabolizing enzymes and oxidative stress in ferric nitrilotriacetate mediated hepatic injury. Redox Report 2:385-92.
29. Lopes G, et al. (2009) Differences in preservation of canine chilled semen using different transport containers. Animal Reproduction Science 112(1):158-63.
30. Levin Y (2011) The role of statistical power analysis in quantitative proteomics. Proteomics 11(12):2565-67.
31. Müller N, et al. (2010) Bolus ingestion of white and green tea increases the concentration of several flavan-3-ols in plasma, but does not affect markers of oxidative stress in healthy non-smokers. Molecular Nutrition and Food Research 54(11):1636-45.
32. Mao JT, et al. (2010) White Tea Extract Induces Apoptosis in Non–Small Cell Lung Cancer Cells: the Role of Peroxisome Proliferator-Activated Receptor-γ and 15-Lipoxygenases. Cancer Prevention Research 3(9):1132-40.
33. Ferrara L, et al. (2001) The distribution of minerals and flavonoids in the tea plant (*Camellia sinensis*). Farmaco 56(5):397-401.
34. Carvalho M, et al. (2010) Green tea: A promising anticancer agent for renal cell carcinoma. Food Chemistry 122(1):49-54.
35. Unachukwu UJ, et al. (2010) White and green teas (*Camellia sinensis var. sinensis*): variation in phenolic, methylxanthine, and antioxidant profiles. Journal of Food Science 75(6):C541-C48.
36. Li Y, et al. (2006) Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. Food Chemistry 96(2):254-60.
37. de Lamirande E and Gagnon C (1995) Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. Human Reproduction 10(suppl 1):15-21.
38. Pryor WA (1991) The antioxidant nutrients and disease prevention-what do we know and what do we need to find out? American Journal of Clinical Nutrition 53(1 Suppl):391S-93S.
39. WHO (1992) WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge University Press, Cambridge.
40. Hilal Y and Engelhardt U (2007) Characterisation of white tea–Comparison to green and black tea. Journal für Verbraucherschutz und Lebensmittelsicherheit 2(4):414-21.
41. Singh BN, et al. (2011) Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. Biochemical Pharmacology 82(12):1807-21.
42. Khan N and Mukhtar H (2007) Tea polyphenols for health promotion. Life sciences 81(7):519-33.

43. Pan T, et al. (2003) Potential therapeutic properties of green tea polyphenols in Parkinson's disease. Drugs & aging 20(10):711-21.
44. Higashi N, et al. (2005) Epigallocatechin-3-gallate, a green-tea polyphenol, suppresses Rho signaling in TWNT-4 human hepatic stellate cells. The Journal of Laboratory and Clinical Medicine 145(6):316-22.
45. Sugiyama A, et al. (2012) Beneficial effects of (-)-epigallocatechin gallate on ischemia-reperfusion testicular injury in rats. Journal of Pediatric Surgery 47(7):1427-32.
46. de Amicis F, et al. (2012) Epigallocatechin gallate affects survival and metabolism of human sperm. Molecular Nutrition and Food Research 56(11):1655-64.
47. Almajano MP, et al. (2008) Antioxidant and antimicrobial activities of tea infusions. Food Chemistry 108(1):55-63.
48. Friedman M (2007) Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. Molecular Nutrition and Food Research 51(1):116-34.
49. Reto M, et al. (2007) Chemical composition of green tea (*Camellia sinensis*) infusions commercialized in Portugal. Plant Foods for Human Nutrition 62(4):139-44.
50. Sugiura C, et al. (2012) Catechins and Caffeine Inhibit Fat Accumulation in Mice through the Improvement of Hepatic Lipid Metabolism. Journal of Obesity 2012:510-20.
51. Baumber J, et al. (2000) The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. Journal of Andrology 21(6):895-902.
52. Tuncer PB, et al. (2010) The effect of raffinose and methionine on frozen/thawed Angora buck (Capra hircus ancryrensis) semen quality, lipid peroxidation and antioxidant enzyme activities. Cryobiology 61(1):89-93.
53. Tremellen K (2008) Oxidative stress and male infertility--a clinical perspective. Human Reproduction Update 14(3):243-58.
54. Silber SJ (1997) The use of epididymal sperm for the treatment of male infertility. Baillière's Clinical Obstetrics and Gynaecology 11(4):739-52.
55. De Pauw I, et al. (2003) In vitro survival of bovine spermatozoa stored at room temperature under epididymal conditions. Theriogenology 59(5):1093-107.
56. Cohen J, et al. (1985) Prolonged storage of human spermatozoa at room temperature or in a refrigerator. Fertility and Sterility 44(2):254-62.
57. Marín-Briggiler CI, et al. (2002) Effect of incubating human sperm at room temperature on capacitation-related events. Fertility and Sterility 77(2):252-59.
58. Vishwanath R and Shannon P (2000) Storage of bovine semen in liquid and frozen state. Animal Reproduction Science 62(1):23-53.
59. Sato M, et al. (2001) Prolonged survival of mouse epididymal spermatozoa stored at room temperature. Genesis 31(4):147-55.
60. Dohle GR, et al. (2005) EAU guidelines on male infertility. European Urology 48(5):703-11.
61. Hull MG, et al. (1985) Population study of causes, treatment, and outcome of infertility. British Medical Journal 291(6510):1693-97.
62. Aitken RJ and Clarkson JS (1987) Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. Journal of Reproduction and Fertility 81(2):459-69.
63. Koppers AJ, et al. (2008) Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. Journal of Clinical Endocrinology and Metabolism 93(8):3199-207.
64. Agarwal A, et al. (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. Fertility and Sterility 79(4):829-43.
65. Gomez E, et al. (1996) Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. Journal of Andrology 17(3):276-87.

Single and synergistic effect of major tea components caffeine, epigallocatechin gallate and L-theanine in rat sperm viability

Abstract

Caffeine, epigallocatechin gallate (EGCG) and L-theanine are major components of tea (*Camellia sinensis* L.) and main representatives of the classes of methylxanthines, catechins and free amino acids. There are many studies reporting the health benefits of tea, however, it is not clear if those effects are mediated by a single component or a synergistic action between them. The aim of this study was to evaluate the individual and synergistic effects of tea's major components in rat epididymal spermatozoa survival and oxidative profile during 3-day storage at room temperature (RT). Spermatozoa were incubated either with caffeine (71 µg/mL), EGCG (82 µg/mL), or L-theanine (19 µg/mL), alone or combined. Spermatozoa viability was assessed by the eosin-nigrosin staining technique. The oxidative profile was established by evaluating the levels of carbonyl groups, protein nitration and lipid peroxidation. Supplementation of sperm storage medium with the three compounds together has improved sperm viability, after 24, 48 and 72 h of incubation, relatively to the control and the groups incubated with each component individually. However, at the end of the 72 h of incubation, there was an increase in protein oxidation in the group exposed to the three compounds, illustrating that the combined treatment triggers different alterations in sperm proteins during their maturational process in the epididymis. This study highlights the importance of the combined effect of all tea components for the beneficial effects usually attributed to tea, particularly in sperm storage at RT.

Keywords: Caffeine; *Camellia sinensis*; epigallocatechin gallate; L-theanine; sperm viability; tea.

Introduction

Tea is one of the most popular beverages in the world, mostly due to its potential health benefits. Four main different types of tea can be obtained from the tea plant (*Camellia sinensis* (L.)), according to the degree of processing to which leaves are submitted: white (less processed), green, oolong or black tea (further processed) [1]. The composition of each type of tea is highly influenced by several factors, including the leaves maturation, processing, geographical origin or botanical variety. Generally, the most bioactive components of tea are caffeine, catechins and L-theanine [2]. Caffeine belongs to the methylxanthines family and is very abundant in coffee beans and tea leaves [3]. Its ingestion has become a daily routine in several people's lives due to its potent stimulant properties [4]. On the other hand, catechins, also known as flavan-3-ols, are the major phenolic compounds present in tea leaves [5]. In this class of phytochemicals, epigallocatechin gallate (EGCG) is the most abundant (50–80% of total

catechins) and the antioxidant activity of tea is associated to its EGCG content [6]. L-theanine is one of the tea's predominant non-protein amino acids, contributing to its distinctive umami taste [7]. Several studies demonstrated the promising actions of these tea components against several human diseases, alone or in combination [1, 8]. However, it remains to be investigated if the health benefits of tea beverage are due to the action of a specific constituent or to a combined action of all constituents.

Concerning male reproductive biology, great efforts are being made to prolong viability of stored spermatozoa as it is a key factor for the success of assisted reproductive technology (ART). It has been reported that room temperature (RT)-storage of spermatozoa for short-term periods could be advantageous, not only to collect sperm from certain species under unexpected conditions, but also to overcome the rapid decline of viability evidenced by spermatozoa when stored in refrigerated environments [9]. This rapid decline has been associated with increased oxidative stress (OS) due to spermatozoa high metabolic rates [9]. Despite several storage media have been tested, the obtained results still show a short-time decrease in sperm viability after incubation at RT [10, 11]. As tea is very rich in antioxidant compounds, we have previously evaluated the effects of tea supplementation on the viability of rat spermatozoa and, interestingly, we observed a great improvement [12]. However, it was not investigated if a single tea compound or a combined action mediated the positive effects detected. Thus, we aimed to evaluate the effects of caffeine, EGCG and L-theanine, individually and in combination, on the survival and oxidative profile of rat spermatozoa during 72 h of storage at RT.

Material and Methods

Chemicals

Tris-base (MB01601) was purchased from NZYTech (Lisbon, Portugal). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specifically stated.

Animal handling and ethical issues

This study was conducted with sperm from one male Wistar rat (*Rattus norvegicus*), having 3-months of age and proven fertility, obtained from our accredited animal colony (Health Sciences Research Center, University of Beira interior). This rat presented normal seminal parameters and enough semen to perform the whole study, and as we support the three R's rule (Reduce, Reuse and Recycle), we found adequate to avoid the unnecessary sacrifice of other animals. The rat was maintained with free access to chow (4RF21 certificate, Mucedola, Italy) and water and maintained under controlled conditions of temperature (20 ± 2°C), humidity (45–65%), air changes/hour (15–20), artificial light/dark cycles (12 h) and noise level (< 55 dB). The study was approved by the local ethical committees and by the Portuguese Veterinarian and Food Department, as required by the Portuguese law (Ordinance no. 1005/92 of 23rd October). All the procedures were performed in accordance to the "Guide for the Care

and Use of Laboratory Animals" (US National Institutes of Health, Publication No. 85-23, revised 1996) and the rules for the care and handling of laboratory animals (Directive 2010/63/EU).

Isolation of epididymal spermatozoa

The rat was anesthetized and killed by decapitation. Both epididymides were immediately removed and placed in a pre-warmed (37°C) plate containing Krebs-Ringer bicarbonate (TYH) medium (118.8 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.56 mM glucose, 1.01 mM sodium pyruvate, sodium 29.2 mM lactate, supplemented with 4.00 mg/mL bovine serum albumin (BSA), 0.06 mg/mL potassium penicillin G and 0.05 mg/mL streptomycin sulfate) prepared on the day of the experiment, as described by Toyoda [13]. This medium is commonly used in cells and tissue cultures to maintain pH and osmotic balance. Both cauda epididymides were gently minced together with a scalpel blade and suspension was left at 37°C for 15 min to allow sperm to disperse into the medium. The remaining epididymal tissue was removed using forceps. The number of spermatozoa was determined using a hemocytometer.

Experimental design

Five experimental groups (n = 6 for each condition) were defined in a 48-well plate, with 1.5 x 10^6 spermatozoa per well. The control group was incubated with a control medium (96.66 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$; 4.15 mM $NaHCO_3$, 5.56 mM D-Glucose, 0.33 mM sodium pyruvate, 23.28 mM sodium lactate, 20.85 hydroxyethyl piperazineethanesulfonic acid (HEPES), supplemented with 4.00 mg/mL BSA, 0.06 mg/mL potassium penicillin G, 0.05 mg/mL streptomycin sulfate) that presents only a few adaptations relative to TYH medium, but has previously demonstrated a higher improvement in spermatozoa survival at RT [9]. The other four groups were exposed to the control medium supplemented with 71 µg/mL of caffeine (C0750, Sigma Aldrich, St. Louis, MO, USA), 82 µg/mL of EGCG (E4143, Sigma Aldrich, St. Louis, MO, USA), 19 µg/mL of L-Theanine (ab141187, Abcam, Cambridge, UK) or the combination of these three compounds (MIX group), respectively. The concentrations were selected according to our previous study reporting the concentrations of these compounds on a WTEA extract for comparative purposes. Sperm suspensions were kept in the dark at acclimatized RT (22-23°C) during 24, 48 and 72 h.

Sperm viability evaluation

Sperm viability was assessed at 0, 24, 48 and 72 hours of incubation by eosin-nigrosin staining technique [12]. In brief, eosin penetrates spermatozoa whose cell membrane integrity is damaged. The number of viable and non-viable spermatozoa was determined counting a total of 100 spermatozoa per slide in continuous random fields under an optical microscope, with oil immersion (x1000 magnification), to determine the percentage of viable sperm, as described [14].

Analysis of protein carbonylation, protein nitration and lipid peroxidation

Spermatozoa were separated from the storage media through centrifugation at 5000 x g for 15 min at 4°C, and total proteins were isolated using Mammalian Protein Extraction Reagent (M-PER) (#89842, Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Protein carbonylation, protein nitration and lipid peroxidation are used as biomarkers for oxidation and can be evaluated by measuring its resulting products, 2,4-dinitrophenol (DNP), nitro-tyrosine and 4-hydroxynonenal (4-HNE) groups, respectively. The content of these adducts was evaluated by the slot-blot technique, as previously described [15]. The resulting polyvinylidene difluoride (PVDF) membranes were incubated overnight (4°C) with rabbit anti-DNP (D9656), rabbit anti-nitro-tyrosine (#9691, Cell signaling Technology, Leiden, Netherlands) or goat anti-4-HNE (AB5605, Merck Millipore Temecula, USA) primary antibodies (dilution 1:5000). The immunoreactive proteins were detected separately and visualized with goat anti-rabbit IgG-alkaline phosphatase (AP) (A3687) or rabbit anti-goat IgG-AP (A4187) at dilution 1:5000. Results were expressed as fold variation to the control.

Statistical analysis

Statistical significance was assessed by one-way ANOVA, followed by Fisher's LSD post-test using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). All data are presented as mean ± SEM from six sperm preparations (n = 6). Differences with $p < 0.05$ were considered statistically significant.

Results and Discussion

As spermatozoa are stored in cauda epididymis in a viable state until ejaculation, epididymal spermatozoa are often used in ART in humans and animals. The maintenance of sperm viability is essential for the preservation of male fertility. In fact, the loss of sperm function is one of the main problems verified in individuals with subfertility or infertility. For that reason, sperm viability is a critical factor in the analysis of sperm quality [16]. Many researchers have been searching for new approaches to store spermatozoa from cauda epididymis, mainly using cryopreservation and refrigeration techniques. However, sperm viability rapidly declines after storage in refrigerated environments and thus, it has been proposed that RT storage for short-term periods can be an effective alternative [10]. Various media have been tested in order to improve spermatozoa survival at RT [9]. Despite the ameliorating effects of certain media, viability is still abruptly decreasing in a short-time [9]. Notably, the addition of tea extracts to sperm RT-storage media has shown promising results. The viability of rat spermatozoa was preserved after exposure to a white or green tea extract during 3 days [12]. Nevertheless, tea composition is diverse, and it was not clear if the detected beneficial effects were due to the action of an individual component or a combined effect. Thus, in this study, we evaluated the effect of the most representative and bioactive components of unfermented teas, either individually or combined, on rat spermatozoa viability after RT storage over a period up to 3

days. Our data demonstrated that at the time of epididymal collection (0 h), sperm viability averaged 62 ± 7%, which was consistent with the results obtained in our previous study [12]. As expected, during spermatozoa RT-storage in control medium, there was a decrease in viability over time (20 ± 3, 16 ± 2 and 13 ± 1%, at 24, 48 and 72 h, respectively) (Figure 4.7). Supplementation of sperm storage medium either with caffeine, EGCG, or L-theanine also led to a decrease in sperm viability during the 3 days of incubation, relative to that observed at the collection time, but not to the control group. Supplementation of spermatozoa with caffeine led to an average viability of 14 ± 1, 17 ± 2 and 11 ± 1% at 24, 48 and 72 h, respectively. Concerning the EGCG group, spermatozoa viability at 24 h was 18 ± 2% and this value was maintained over the 48 h (17 ± 1%) and 72 h (17 ± 3%). Spermatozoa supplemented with L-theanine showed a viability of 20 ± 3% at 24 h, 22 ± 4% at 48 h and 11 ± 2% at 72 h. These results illustrate that, at the used doses, the individual addition of such compounds to the sperm storage medium do not improve rat epididymal spermatozoa survival. Considering these results and those obtained in our previous study, which evidenced a beneficial effect of supplementing sperm storage medium with tea extracts, we can affirm that none of these components is individually responsible for the observed improvement in the viability of spermatozoa incubated with tea [12]. Nevertheless, the incubation of spermatozoa in the medium containing a mixture of caffeine, EGCG and L-theanine also led to a decreased amount of viable sperm when compared with collection time (0 h) and a higher viability when compared to the control group at 24, 48 and 72 h. Moreover, at 24 h, spermatozoa from the MIX group demonstrated a higher viability (32 ± 3%) when compared to the groups incubated with each compound individually (Figure 4.7).

At 48 h, the same was verified and sperm viability in the MIX group achieved 35 ± 6%, being higher than the viability evidenced by the groups incubated with caffeine or EGCG. Finally, at 72 h there was a decrease in viability to 24 ± 2% in spermatozoa of the MIX group, though this value was higher than in groups incubated with caffeine or L-theanine. Notably, despite the improvement on sperm viability induced by the MIX, comparatively to each compound individually, it was less significant than that reported with supplementation with the tea extract [12]. Thus, our results provide compelling evidence that the beneficial effects of tea extract in rat sperm survival at RT, results from a combined effect of several tea components, far beyond the three major bioactive components caffeine, EGCG and L-theanine. Nevertheless, the combined effects of these compounds are more effective than the verified individually.

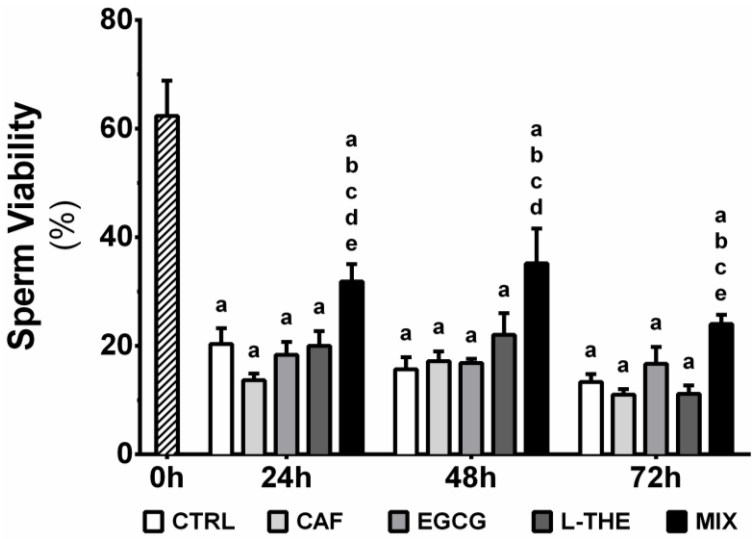


Figure 4.7 Spermatozoa viability at collection time (0 h) and during the 3-day storage in control medium (CTRL) and media supplemented with caffeine (CAF), epigallocatechin gallate (EGCG), L-theanine (L-THE) or the combination of the three compounds (MIX). Results are expressed in percentage and presented as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as: a – relative to 0 h; b - relative to control; c - relative to CAF; d - relative to EGCG; e - relative to L-THE.

Spermatozoa are highly dependent on exogenous substrates and present high metabolic rates, which are often associated with an elevated production of reactive oxygen species (ROS). While small amounts of ROS are required for normal sperm functioning, unbalanced ROS production is one of the main factors responsible for poor semen quality as it may lead to cellular damages [17-19]. In fact, ROS can induce the production of carbonyl derivatives and the formation of nitro groups in proteins, as well as the degradation of lipids from the cells membranes (lipid peroxidation) [15]. The decline on spermatozoa viability is associated with increased levels of OS, thus the control of ROS production is crucial to preserve sperm quality. We have previously shown that supplementation of spermatozoa storage medium with tea extracts, which possess a potent antioxidant potential, was able to decrease lipid peroxidation in spermatozoa after 72 h [12]. Considering the antioxidant properties usually ascribed to tea components, we hypothesized that caffeine, EGCG and L-theanine, individually or mixed, could have a beneficial effect on the maintenance of spermatozoa oxidative status during its storage at RT. Yet, no alterations were found concerning the levels of protein nitration and lipid peroxidation between the experimental groups (Figure 4.8). Spermatozoa present a high content in polyunsaturated fatty acids (PUFAs) in their plasma membrane, which confers them the fluidity that is required for motility acquisition. However, this high content in PUFAs, together with the lack of the necessary cytoplasmic-enzyme repair systems in spermatozoa, makes them particularly susceptible to lipid peroxidation [20]. In our previous study, we have associated the increase in the antioxidant potential of sperm storage media after supplementation with tea extracts and the decrease in sperm lipid peroxidation with the verified improvements in sperm viability [12]. However, in this study, sperm lipid peroxidation remained unaltered after exposure to the tea compounds, alone or in combination, suggesting that these compounds do not directly

modulate that mechanism in the tested conditions. Similarly, no changes were observed in protein nitration of epididymal spermatozoa stored in any of the experimental media. This was an expected result because protein nitration is usually associated with the process of sperm capacitation [21], which starts on epididymis but is only completed in the female reproductive tract. Still, there was an increase in carbonyl groups levels in the group of spermatozoa supplemented with the MIX (1.4 ± 0.1-fold variation to the control) in comparison with the control and the groups supplemented individually either with caffeine (1 ± 0.1-fold variation to the control) or L-theanine (1 ± 0.1-fold variation to the control) (Figure 4.8). In normal conditions, sperm maturational processes during epididymis transit involve an extensive remodeling of proteins from the plasma membrane that generally generates a controlled amount of ROS [22]. Our results suggest that the combined action of caffeine, EGCG and L-theanine may interfere with these remodeling processes in sperm proteins, leading to the observed increase in carbonyl groups levels that indicates protein oxidation.

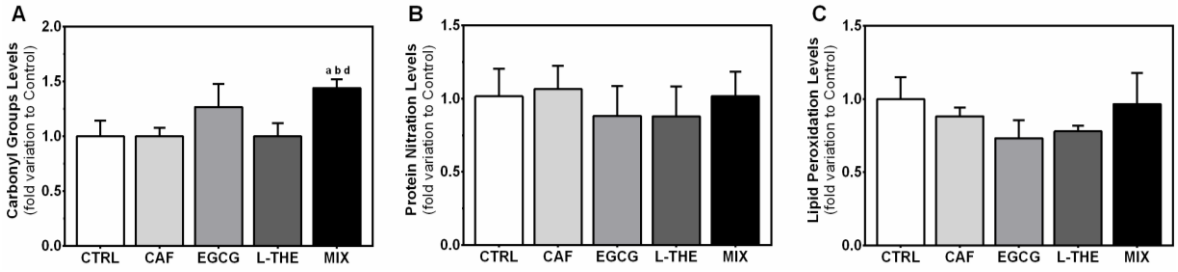


Figure 4.8 Spermatozoa oxidative profile at the end of incubation in control medium (CTRL) and media supplemented with caffeine (CAF), epigallocatechin gallate (EGCG), L-theanine (L-THE) or the combination of the three compounds (MIX). The figure shows pooled data of independent experiments, indicating carbonyl group levels (Panel A), protein nitration (Panel B) and lipid peroxidation (Panel C). Results are presented as fold variation to control and expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as: a - versus 0 h; b - versus control; c - versus CAF; d - versus EGCG.

In summary, this study provides evidence for a beneficial effect of the combination of caffeine, EGCG and L-theanine in rat sperm survival at RT, representing a further step in clarifying the tea components responsible for the promising benefits previously reported to a supplementation with white tea extract in spermatozoa storage medium. Our results highlight the relevance of the combined action of all tea constituents, although not yet completely understood, being concurrent with previous reports that show different effects of tea compounds either alone or combined [8]. Additional studies will be needed to disclose the molecular mechanisms by which natural products, particularly tea, can contribute with specific compounds or a mixture of phytochemicals to improve spermatozoa preservation and male fertility. Still, we can infer that the beneficial effects usually attributed to tea are not induced by its major bioactive compounds, but rather by the combined action of all compounds.

References

1. da Silva Pinto M (2013) Tea: A new perspective on health benefits. Food Research International 53(2):558-67.
2. de Mejia EG, et al. (2009) Bioactive components of tea: cancer, inflammation and behavior. Brain, behavior, and immunity 23(6):721-31.
3. Dias TR, et al. (2015) Dose-dependent effects of caffeine in human Sertoli cells metabolism and oxidative profile: Relevance for male fertility. Toxicology 328:12-20.
4. Glade MJ (2010) Caffeine—not just a stimulant. Nutrition 26(10):932-38.
5. Dias TR, et al. (2013) White tea (Camellia sinensis (L.)): antioxidant properties and beneficial health effects. International Journal of Food Science, Nutrition and Dietetics 2(2):19-26.
6. Khan N and Mukhtar H (2007) Tea polyphenols for health promotion. Life sciences 81(7):519-33.
7. Vuong QV, et al. (2011) L-Theanine: properties, synthesis and isolation from tea. Journal of the Science of Food and Agriculture 91(11):1931-39.
8. Zheng G, et al. (2004) Anti-obesity effects of three major components of green tea, catechins, caffeine and theanine, in mice. In Vivo 18(1):55-62.
9. Sato M and Ishikawa A (2004) Room temperature storage of mouse epididymal spermatozoa: exploration of factors affecting sperm survival. Theriogenology 61(7):1455-69.
10. Sato M, et al. (2001) Prolonged survival of mouse epididymal spermatozoa stored at room temperature. genesis 31(4):147-55.
11. Marín-Briggiler CI, et al. (2002) Effect of incubating human sperm at room temperature on capacitation-related events. Fertility and sterility 77(2):252-59.
12. Dias TR, et al. (2014) White Tea as a Promising Antioxidant Medium Additive for Sperm Storage at Room Temperature: A Comparative Study with Green Tea. Journal of agricultural and food chemistry 62(3):608-17.
13. Toyoda Y (1971) Studies on fertilization of mouse eggs in vitro. I. In vitro fertilization of eggs by fresh epididymal sperm. The Japanese journal of animal reproduction 16(4):147-51.
14. Rato L, et al. (2013) High-energy diets may induce a pre-diabetic state altering testicular glycolytic metabolic profile and male reproductive parameters. Andrology 1(3):495–504.
15. Dias TR, et al. (2015) Dehydroepiandrosterone and 7-oxo-dehydroepiandrosterone in male reproductive health: implications of differential regulation of human Sertoli cells metabolic profile. The Journal of Steroid Biochemistry and Molecular Biology 154:1-11.
16. Rato L, et al. (2015) Testicular Metabolic Reprogramming in Neonatal Streptozotocin-Induced Type 2 Diabetic Rats Impairs Glycolytic Flux and Promotes Glycogen Synthesis. Journal of Diabetes Research 2015:973142.
17. Dias TR, et al. (2014) Sperm glucose transport and metabolism in diabetic individuals. Molecular and cellular endocrinology 396(1-2):37-45.
18. Latchoumycandane C, et al. (2002) Induction of oxidative stress in rat epididymal sperm after exposure to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin. Archives of toxicology 76(2):113-18.
19. Latchoumycandane C, et al. (2002) The effect of methoxychlor on the epididymal antioxidant system of adult rats. Reproductive Toxicology 16(2):161-72.
20. Baumber J, et al. (2000) The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. Journal of andrology 21(6):895-902.
21. de Lamirande E and Lamothe G (2009) Reactive oxygen-induced reactive oxygen formation during human sperm capacitation. Free Radical Biology and Medicine 46(4):502-10.
22. Cuasnicú P, et al. (2002) Changes in Specific Sperm Proteins During Epididymal Maturation. In: Robaire B and Hinton B (eds) The Epididymis: From Molecules to Clinical Practice, Springer US, pp. 389-403

Chapter 5

III - In Vivo Study

This chapter was adapted from the following publication:

a) **Dias TR**, Alves MG, Rato L, Casal S, Silva BM, Oliveira PF. (2016) "White tea intake prevents prediabetes-induced metabolic dysfunctions in testis and epididymis preserving sperm quality" The Journal of Nutritional Biochemistry 37:83-93 (DOI:10.1016/j.jnutbio.2016.07.018).

White tea intake prevents prediabetes-induced metabolic dysfunctions in testis and epididymis preserving sperm quality

Abstract

Prediabetes has been associated with alterations in male reproductive tract, especially in testis and epididymis. I*n vitro* studies described a promising action of tea (*Camellia sinensis* L.) against metabolic dysfunctions. Herein, we hypothesized that white tea (WTEA) ingestion by prediabetic rats could prevent the metabolic alterations induced by the disease in testicular and epididymal tissues, preserving sperm quality. WTEA infusion was prepared and its phytochemical profile was evaluated by proton nuclear magnetic resonance (^{1}H-NMR). A streptozotocin-induced prediabetic rat model was developed and three experimental groups were defined: control, prediabetic (PreDM) and prediabetic drinking WTEA (PreDM+WTEA). Metabolic profile of testes and epididymides was evaluated by determining the metabolites content (^{1}H-NMR), protein levels (western blot) and enzymatic activities of key metabolic intervenient. The quality of spermatozoa from cauda epididymis was also assessed. Prediabetes increased glucose transporter 3 (GLUT3) protein levels and decreased lactate dehydrogenase (LDH) activity in testes, resulting in a lower lactate content. WTEA ingestion maintained testicular lactate content to normal values. Concerning epididymis, prediabetes decreased the protein levels of several metabolic intervenient, resulting in decreased lactate and alanine content. WTEA consumption prevented most of the epididymal alterations observed in PreDM group, however, lactate content was also reduced. The consumption of WTEA by prediabetic rats also improved epididymal sperm motility. Prediabetes strongly affected testicular and epididymal metabolic status and most of these alterations were prevented by WTEA consumption, resulting in the improvement of sperm quality. Our results suggest that WTEA consumption can be a cost-effective strategy to improve prediabetes-induced reproductive dysfunction.

Keywords: epididymis; prediabetes; spermatogenesis; sperm quality; testis; white tea.

Introduction

Within the testes, spermatogenesis is the process that ensures the development of spermatozoa [1]. It is mostly regulated by Sertoli cells (SCs) [2], which are responsible for the production of the seminiferous tubular fluid (STF) and control of its composition [3]. After being produced, spermatozoa are transported to the epididymis using STF as vehicle. In the epididymis, spermatozoa undergo a maturation process as they migrate through its several regions: caput, corpus and cauda epididymis. Each segment creates a unique microenvironment that allows the spermatozoa to acquire the ability to move spontaneously and to fertilize until they are stored in the cauda epididymis [4]. Metabolic pathways play a crucial role in these processes. In fact,

glucose is an essential substrate not only for testicular and epididymal function, but also for spermatozoa development and quality [5, 6]. Typically, the uptake of glucose derived from carbohydrate metabolism by cells occurs through membrane-specific glucose transporters (GLUTs) [7]. GLUT1, GLUT2 and GLUT3 are the main isoforms found in testis and epididymis [8]. In general, most of glucose is converted to pyruvate in a rate-limiting process catalyzed by the enzyme phosphofructokinase 1 (PFK1). Pyruvate can be further converted into lactate or alanine by the action of lactate dehydrogenase (LDH) or alanine transaminase (ALT), respectively. Besides, pyruvate can enter the mitochondria to fuel the Krebs cycle [7]. In the testes, lactate present in intratubular fluid is exported by the specific monocarboxylate transporter 4 (MCT4) to be used by germ cells [9]. On the other hand, alanine plays a key role in the maintenance of cellular redox status and glucose homeostasis [10].

Under normal physiological conditions, glucose metabolism is strictly controlled [11]. However, diabetic patients have a dysregulation of glucose metabolism that might lead to increased accumulation of this hexose in the blood (hyperglycemia), which has long been associated with male reproductive dysfunction [12, 13]. In fact, it has been reported that 35% of the male patients with type 2 diabetes *mellitus* (T2DM), the most prevalent type of diabetes *mellitus* (DM), have some form of subfertility and/or infertility [14]. Severe metabolic alterations and decreased sperm quality are usually found in individuals with T2DM [11]. More recently, the prodromal stage of T2DM [15], prediabetes, has also been associated with alterations in male reproductive health [15]. Prediabetes is characterized by resistance to an insulin-mediated glucose disposal and a compensatory hyperinsulinemia [16]. It has been estimated that 10% of the cases of prediabetes progress to T2DM every year [17]. Prediabetes was also associated with alterations in testicular metabolic profile and impairment of sperm parameters [18]. Recently, it was reported that prediabetes affects the membrane transport systems in testis and epididymis [19, 20]. Therefore, it is important to counteract the metabolic alterations induced by this disease and prevent its associated reproductive complications.

Lifestyle modifications usually constitute the preferential treatment for prediabetes, however, in most severe cases, antidiabetic drugs need to be prescribed [21]. For instance, the antidiabetic metformin, besides preventing the progression of prediabetes into T2DM, also modulates testicular cell metabolism [11, 22, 23]. However, side effects such as gastrointestinal irritation, lactic acidosis, diarrhea, nausea, vomiting and increased flatulence have been reported [24, 25], which decreases patient's compliance. Although being used since ancient times, natural products are increasingly regarded as a complementary or alternative therapy against several diseases [26-28]. Many natural products, including tea, showed hypoglycemic properties [29-33]. *In vitro* studies suggest that tea catechins help controlling hyperglycemia by enhancing insulin activity and possibly preventing damage to pancreatic ß-cells [34]. White tea (WTEA) is one of the less processed and less studied teas. Besides, it presents higher catechin content than other tea types, which consequently confers it greater antioxidant activity [35]. We have previously demonstrated that the addition of a WTEA extract

to an epididymal spermatozoa storage medium strongly improves sperm viability [36]. Furthermore, the exposure of rat cultured SCs to a WTEA extract led to favorable alterations in cell glycolytic profile, as there was a stimulation in lactate production [37]. Recently, daily consumption of WTEA by prediabetic Wistar rats improved testicular oxidative profile and epididymal sperm quality [38]. Herein, we hypothesize that WTEA ingestion could ameliorate the negative metabolic effects induced by the disease in testes and epididymis, preserving sperm quality in prediabetic male rats.

Materials and Methods

Chemicals

NZYColour Protein Marker II was purchased from NZYTech (Lisbon, Portugal). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specifically stated.

White tea aqueous infusion

WTEA infusion was prepared daily from a commercial brand (Diese, Bestlife - Comércio e Distribuição Lda., Portugal) according to the manufacturer's instructions. Briefly, samples were subjected to infusion (1 g / 100 mL of distilled boiling water) during 3 min. The resulting infusion was filtered with qualitative filter papers (Cat. No. 516-0819, VWR, Leuven, France) in a vacuum system.

Animal handling and ethical issues

Eighteen male Wistar rats (*Rattus norvegicus*) were obtained from our accredited animal colony (Health Sciences Research Center, University of Beira interior). All animals were housed in type III-H cages (Tecniplast, Italy) and maintained under controlled conditions of temperature (20 ± 2°C), humidity (45–65%), air changes/hour (15–20), artificial light/dark cycles (12 h) and noise level (< 55 dB). The study was approved by the local ethical committees and by the Portuguese Veterinarian and Food Department, as required by the Portuguese law (Ordinance no. 1005/92 of 23rd October). All the procedures followed were in accordance to the "Guide for the Care and Use of Laboratory Animals" (US National Institutes of Health, Publication No. 85-23, revised 1996) and the rules for the care and handling of laboratory animals (Directive 2010/63/EU).

Experimental approach

Firstly, male Wistar rats were randomly distributed into a control (n=6) and PreDM (n=12) groups. All animals had free access to chow (4RF21 certificate, Mucedola, Italy) and water. The available chow was composed by: 66.5% cereals; 18.2% vegetable protein (soybean meal and yeast); 7.5% forage; 3.5% animal protein; 3.2% vitaminic and mineral mixture; 0.4% fats (soya oil) and 0.1% amino acids. To achieve the prediabetic model, animals from PreDM group, which weighted about 10 g, were injected with a low-dose of streptozotocin (STZ) (40 mg / kg of body weight) at two days of age, as previously described [38, 39]. Briefly, after an eight-hour fasting,

STZ was freshly diluted in citrate buffer (0.1 M, sodium citrate, pH 4.5) and injected intraperitoneally in the animals. Rats from the control group only received the vehicle solution in an equivalent volume. At 1-month-old, six of the animals of the PreDM group were randomly selected and assigned to the PreDM+WTEA (n=6) group. After the establishment of the three experimental groups (n=6 for each group), no differences were found in animal's body weight. Animals had free access to food and water for two months. Though, in the PreDM+WTEA group water was replaced by WTEA infusion. Blood glucose levels of all animals were weekly monitored from the first to the third month of age using a glucometer (One Touch Ultra Lifescan-Johnson, Milpitas, CA, USA). At 3 months of age, all animals were submitted to a glucose tolerance test (GTT) and an insulin tolerance test (ITT), as described previously [38, 40]. Blood was collected from the tail vein and glucose levels were measured right before the injection of glucose/insulin and after 30, 60, 90 and 120 min. Subsequently, animals were anesthetized and killed by decapitation. Rats were sacrificed between 9:00 AM and 11:00 AM to avoid the effects of diurnal variation. We have chosen the 3-month experimental period to assure that the animals had attained a mature reproductive function. Despite reaching sexual maturity at approximately 38 days of age, rats only reach puberty around 50 days of age [41]. As the duration of rat spermatogenic cycle is about 13 days [42], at 3-months of age, rats would have completed at least 2 spermatogenic cycles, thus being considered as animals with fully mature reproductive function. After the sacrifice, both testes and one cauda epididymis of each animal were immediately removed and stored at -80°C for further analysis, while the other cauda epididymis was placed in 3 mL of Hanks Balanced Salt Solution (HBSS; pH 7.4), preheated at 37°C, to be used for sperm quality analysis.

Proton nuclear magnetic resonance spectroscopy

Testicular and epididymal tissue extracts were prepared using a combined extraction of polar and apolar metabolites as previously described [7]. In brief, tissue was homogenized in a mixture of methanol and chloroform (2:1). Then, a combination of chloroform and water (1:1) was added and samples were centrifuged at 10000 x g for 15 min at 4°C. The resulting supernatant, as well as a sample of WTEA infusion, were lyophilized in a Freeze-Dryer (Labogene, Lynge, Denmark) and further dissolved in deuterium oxide (D_2O) for proton nuclear magnetic resonance (^{1}H-NMR) analysis. ^{1}H-NMR spectra of WTEA and testicular/epididymal tissue extracts were acquired at 14.1 T, 25°C, using a Bruker Avance 600 MHz spectrometer equipped with a 5 mm QXI probe and a z-gradient. The parameters of the acquisition were: a solvent suppression and a sweep width of 6 kHz, a delay of 14 s, a water presaturation of 3 s, a pulse angle of 45°, an acquisition time of 3.5 s, and at least 128 scans. Sodium fumarate (final concentration of 1 mM) was used as internal reference (6.50 ppm) to quantify the following metabolites present in solution (multiplet, ppm): L-theanine (triplet, 1.08); lactate (doublet, 1.33); alanine (doublet, 1.45); acetate (singlet, 1.90); epigallocatechin gallate (EGCG; doublet, 2.7); caffeine (singlet, 3.29); α-glucose (doublet, 5.22); sucrose (doublet, 5.4); epigallocatechin (EGC; singlet, 6.6); epicatechin (EC; singlet, 7.0). The relative areas of ^{1}H-

NMR resonances were quantified using the curve-fitting routine supplied with the NUTSproTM NMR spectral analysis program (Acorn, NMR Inc., Fremont, CA, USA).

Western blot

Total proteins from rat testis and cauda epididymis were isolated using a Lysis buffer (250 mM Sacarose, 20 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), 10 mM KCl, 1.5 mM MgCl2, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), supplemented with 1% protease inhibitor cocktail, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF); pH 7.4). Western blot was performed as previously described [43]. In brief, proteins were fractionated in 12% polyacrylamide gels, then the separated proteins were transferred to previously activated polyvinylidene difluoride (PVDF) membranes and blocked for 90 min with a 5% non-fat milk solution at room temperature (RT). Afterwards, the membranes were incubated overnight at 4°C with the primary antibodies listed in Table 5.1. Mouse anti-α-tubulin was used as the protein loading control. The immunoreactive proteins were detected separately and visualized after incubation with the respective secondary antibodies (Table 5.1) for 90 min, at RT. Membranes were reacted with ECF™ substrate (#RPN5785, GE Healthcare, Buckinghamshire, UK) and read with the BioRad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK). Densities from each band were obtained with BIO-PROFIL Bio-1D Software from Quantity One (Vilber Lourmat, Marne-la-Vallée, France) according to standard methods [42]. The band density attained was divided by the corresponding tubulin band intensities and expressed in fold change to control.

Phosphofructokinase 1 enzymatic assay

PFK1 activity was determined by a fluorometric method based on the conversion of fructose 6-phosphate and adenosine triphosphate (ATP) to fructose 1,6-biphosphate and adenosine diphosphate (ADP), coupled with the conversion of ADP to adenosine monophosphate (AMP) and nicotinamide adenine dinucleotide reduced (NADH) in the presence of aldolase, α-glycerophosphate dehydrogenase and triosephosphate isomerase. While NADH is capable of fluorescent emission at 450 nm, when excited at 340 nm, its oxidized form (NAD^+) is not [7]. Thus, NADH oxidation was followed by the decrease in fluorescence at 30°C. The attained activities were expressed as units (nmol/min) per milligram of protein.

Lactate dehydrogenase enzymatic assay

LDH activity levels were spectrophotometrically determined using a LDH enzymatic assay kit (#88954, Thermo Scientific, Waltham, MA, USA) as previously described [44]. Absorbance was measured at 490 nm using an Anthos 2010 microplate reader (Biochrom, Berlin, Germany). The method was calibrated with LDH positive control included in the assay kit. LDH enzymatic activities were calculated as units (nmol/min) per milligram of protein, using the molar extinction coefficient (ε) of formazan.

Table 5.1 List of the primary and secondary antibodies used in this study.

Antibody	Source	KDa	Dilution	Vendor	Catalog #
GLUT1	Rabbit	55	1:200	Santa Cruz Biotechnology Heidelberg, Germany	sc-7903
GLUT2	Rabbit	60-62	1:5000	Santa Cruz Biotechnology Heidelberg, Germany	sc-9117
GLUT3	Goat	48-70	1:200	Santa Cruz Biotechnology Heidelberg, Germany	sc-7582
PFK1	Rabbit	85	1:500	Santa Cruz Biotechnology Heidelberg, Germany	sc-67028
MCT4	Rabbit	43	1:1000	Santa Cruz Biotechnology Heidelberg, Germany	sc-50329
LDH	Rabbit	37-38	1:10000	Abcam Cambridge, MA, USA	ab52488
ALT	Rabbit	48	1:500	Santa Cruz Biotechnology Heidelberg, Germany	sc-99088
α-Tubulin	Mouse	55	1:5000	Thermo Fisher Scientific Rockford, IL, USA	MA5-16308
Mouse	Goat	—	1:5000	Sigma-Aldrich St. Louis, MO, USA	A3562
Rabbit	Goat	—	1:5000	Sigma-Aldrich St. Louis, MO, USA	A3687
Goat	Rabbit	—	1:5000	Sigma-Aldrich St. Louis, MO, USA	A4187

Abbreviations: GLUT1, glucose transporter 1; GLUT2, glucose transporter 2; GLUT3, glucose transporter 3; PFK1, phosphofructokinase 1; MCT4, monocarboxylate transporter 4; LDH, lactate dehydrogenase; ALT, alanine transaminase.

Epididymal sperm quality evaluation

One cauda epididymis from each animal was minced using scissors to allow the dispersion of spermatozoa into the medium and residual epididymal tissue was removed. The sperm suspension was used to evaluate sperm concentration, motility, viability and morphology as previously described [38, 45].

Statistical analysis

Statistical significance was assessed by one-way ANOVA, followed by Bonferroni post-test using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). All data are presented as mean ± SEM. Relationships between lactate production, LDH protein levels and LDH activity were evaluated using Pearson's correlation coefficient. Differences with $p < 0.05$ were considered statistically significant.

Results

Phytochemical profile of white tea aqueous infusion

The analysis of ^{1}H-NMR spectra allowed the quantitative phytochemical characterization of the WTEA aqueous infusion. The concentration of each compound in the prepared WTEA infusion was calculated (Table 5.2). The most abundant compound was caffeine (0.93 ± 0.10 mM), which belongs to the methylxanthines family and is widely known by its stimulant properties. The WTEA infusion was also very rich in catechin derivatives, with this family of phytochemicals representing a total concentration of 0.87 mM. Three different catechins were found in the

infusion: EC (0.04 ± 0.007 mM); EGC (0.39 ± 0.03 mM) and EGCG (0.44 ± 0.04 mM). Moreover, two carbohydrates were present: sucrose (0.44 ± 0.03 mM) and glucose (0.08 ± 0.01 mM). Two amino acids, L-theanine (0.28 ± 0.03 mM) and alanine (0.02 ± 0.002 mM), were also found. Finally, a low concentration of one monocarboxylic acid, lactate (or lactic acid), was found in the WTEA infusion (0.01 ± 0.001 mM) (Table 5.2).

Table 5.2 White tea infusion phytochemical characterization as determined by proton nuclear magnetic resonance.

Compound	Concentration (mM)
Alanine	0.02 ± 0.002
Caffeine	0.93 ± 0.10
EC	0.04 ± 0.007
EGC	0.39 ± 0.03
EGCG	0.44 ± 0.04
Glucose	0.08 ± 0.01
Lactate	0.01 ± 0.001
L-Theanine	0.28 ± 0.03
Sucrose	0.44 ± 0.03

Abbreviations: EC, epicatechin; EGC, epigallocatechin; EGCG, epigallocatechin gallate. Results are presented as mean ± SEM (n = 5).

Streptozotocin-treated rats developed characteristics of prediabetes which were prevented by white tea consumption

At 3-months of age there were no differences in the body weight of the rats of the different experimental groups. Animals from the control group presented an average weight of 347 ± 8 g, while animals from PreDM and PreDM+WTEA groups weighted in average 363 ± 11 g and 378 ± 13 g, respectively. At the 60th day of treatment, rats from the control group evidenced a blood glycemia of 83.8 ± 1.8 mg/dL. Glycemic levels of animals from PreDM (112.0 ± 1.9 mg/dL) and PreDM+WTEA (111.2 ± 2.0 mg/dL) groups were higher relative to the control (Table 5.3). These results were the first evidence that STZ-treated rats developed prediabetes. According to the "standards of medical care in diabetes" reported by the American Diabetes Association, blood glucose levels between 100 and 125 mg/dL are one of the indicators of prediabetes [46]. We have also performed a GTT and ITT. The shift in blood glucose levels after the GTT was 48.2 ± 8.6 mg/dL in the control group. There was an increase in GTT variation to 114.8 ± 21.1 mg/dL in the PreDM group. However, the PreDM+WTEA group showed a GTT variation similar to the control group (58.8 ± 18.5 mg/dL) (Table 5.3). After performing the ITT, the shift in blood glucose levels observed in the control group was -48.7 ± 11.1 mg/dL. There was a decrease in this ITT shift to -5.3 ± 8.1 mg/dL in the PreDM group. Moreover, there was a decrease in ITT variation in the PreDM+WTEA group (-46.5 ± 12.4 mg/dL) relative to the PreDM group (Table 5.3), but not relative to the control. Thus, STZ-treated animals demonstrated mild hyperglycemia, glucose intolerance and insulin resistance. The PreDM+WTEA group did not show any alterations in glucose tolerance and insulin resistance relative to the control group.

Table 5.3 Effect of white tea consumption on blood glucose levels in prediabetic rats, at the end of the treatment (non-fasting glucose) and after performing the glucose and insulin tolerance tests.

Blood glucose levels (mg/dL)	Control	PreDM	PreDM+WTEA
NFG	83.8 ± 1.8	112 ± 1.9 *	111.2 ± 2.0 *
GTT	48.2 ± 8.6	114.8 ± 21.1 *	58.8 ± 18.5
ITT	-48.7 ± 11.1	-5.3 ± 8.1 *	-46.5 ± 12.4 #

Results are presented in mg/dL and expressed as mean ± SEM (n = 6 for each condition). Results of glucose tolerance test (GTT) and insulin tolerance test (ITT) were obtained by the variation between the blood glucose levels at the end and before of the test. Significantly different results ($p < 0.05$) are indicated as relatively to: * control or # PreDM. NFG, non-fasting glucose; PreDM, prediabetic rats drinking water; PreDM+WTEA, prediabetic rats drinking white tea.

Testicular metabolism

Prediabetic rats drinking white tea showed increased testicular GLUT2 and GLUT3 protein levels and increased PFK1 activity

To evaluate testicular metabolism, we started by studying its main substrate – glucose, which is taken up by testicular cells through GLUTs. No differences were found in GLUT1 protein levels among the experimental groups (Figure 5.1a). Similarly, GLUT2 protein levels were not altered in PreDM group (1.03 ± 0.10-fold change vs control) in comparison to the control group. However, there was an increase in testicular GLUT2 protein levels in PreDM+WTEA group to 1.14 ± 0.09-fold change vs control (Figure 5.1b). GLUT3 protein levels increased in testis of PreDM (1.33 ± 0.10- fold change vs control) and PreDM+WTEA (1.48 ± 1.17-fold change vs control) groups relative to the control (Figure 5.1c).

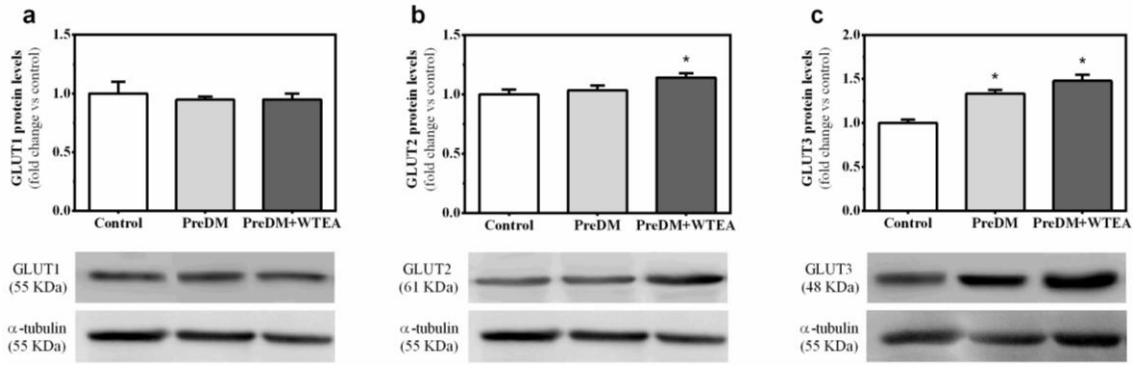


Figure 5.1 Effect of white tea (WTEA) consumption in testicular glucose transport of prediabetic rats. The figure shows pooled data of independent experiments, indicating glucose transporters, GLUT1 (a), GLUT2 (b) and GLUT3 (c) protein levels in control, prediabetic rats drinking water (PreDM) or white tea (PreDM+WTEA). The representative blots (of one sample per each condition) of GLUT1, GLUT2 and GLUT3 are also shown. Variation in protein levels is presented as fold change vs control. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as relatively to: * control.

After glucose enters the testis, there is a major rate-limiting step in the glycolytic pathway that is catalyzed by PFK1 and results in pyruvate production. The ingestion of WTEA by prediabetic rats led to decreased PFK1 protein levels (0.78 ± 0.10-fold change vs control) when compared to animals from control and PreDM (1.08 ± 0.13-fold change vs control) groups (Figure 5.2a). However, PFK1 activity has increased about 2-fold in the group of prediabetic animals drinking WTEA (10.9 ± 1.4 nmol/min/mg protein) relative to control (5.8 ± 0.3 nmol/min/mg protein) and PreDM (5.6 ± 0.4 nmol/min/mg protein) groups (Figure 5.2b).

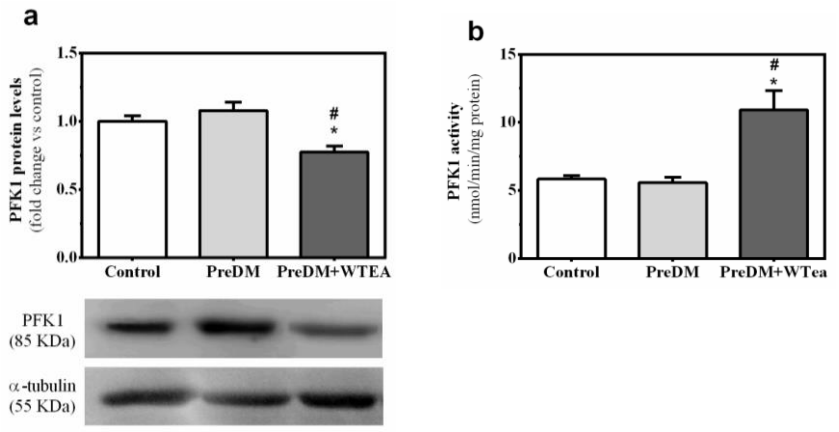


Figure 5.2 Effect of white tea (WTEA) consumption in testicular glycolytic profile of prediabetic rats. The figure shows pooled data of independent experiments, indicating phosphofructokinase 1 (PFK1) protein levels (a) and activity (b) in control, prediabetic rats drinking water (PreDM) or white tea (PreDM+WTEA). The representative blots (of one sample per each condition) of PFK1 are also shown. Variation in protein levels is presented as fold change vs control. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as relatively to: * control or # PreDM.

Testicular lactate content was decreased in prediabetic rats but not in prediabetic rats drinking white tea

Testicular pyruvate is mainly converted to lactate by LDH. While no alterations were found in testicular LDH protein levels among the experimental groups (Figure 5.3a), there was a decrease in LDH activity to 77.1 ± 3.7 nmol/min/mg protein in the PreDM group (Figure 5.3b), relative to the control group (93.3 ± 2.9 nmol/min/mg protein). Accordingly, testicular lactate content decreased to 1543 ± 311 pmol/mg_{ww} in testis of PreDM group in comparison to control animals (3030 ± 487 pmol/mg_{ww}) (Figure 5.3c). The ingestion of WTEA by prediabetic rats also induced a decrease in LDH activity (60.8 ± 2.0 nmol/min/mg protein) not only relative to the control, but also to the PreDM group (Figure 5.3b). Nevertheless, prediabetic rats drinking WTEA did not show alterations in testicular lactate content when compared to the control group (2274 ± 234 pmol/mg_{ww}) (Figure 5.3c).

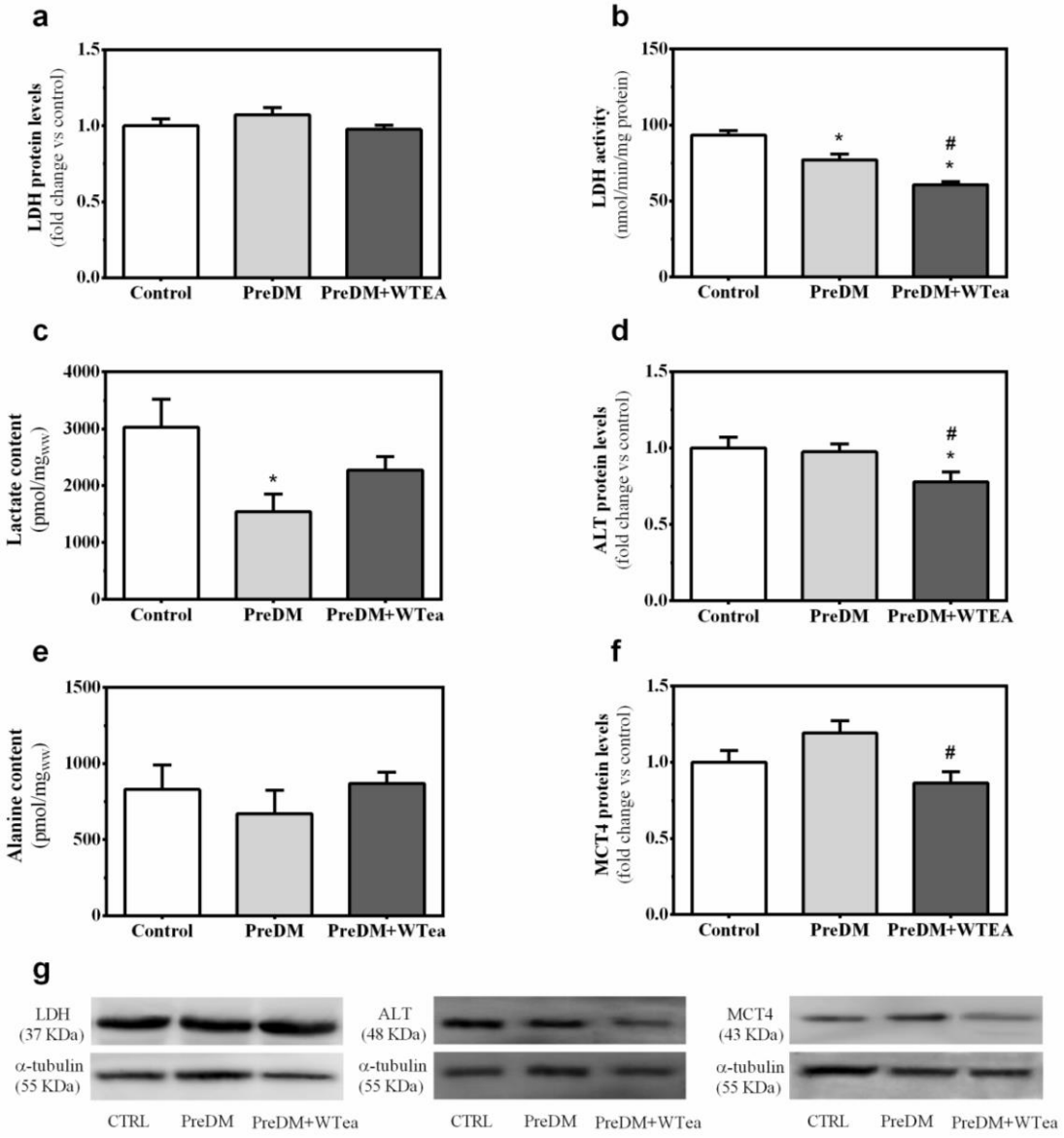


Figure 5.3 Effect of white tea (WTEA) consumption in testicular content of lactate and alanine in prediabetic rats. The figure shows pooled data of independent experiments, indicating lactate dehydrogenase (LDH) protein levels (a), LDH activity (b) and lactate content (c) in control, prediabetic rats drinking water (PreDM) or white tea (PreDM+WTEA). The figure also shows alanine transaminase (ALT) protein levels (d), alanine content (e) and monocarboxylate transporter 4 (MCT4) protein levels (f). The representative blots (of one sample per each condition) of LDH, ALT and MCT4 are also shown (g). Variation in protein levels is presented as fold change vs control. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as relatively to: * control or # PreDM.

White tea ingestion by prediabetic rats led to decreased testicular protein levels of ALT but maintained alanine content

Besides being mainly used to produce lactate, pyruvate derived from glycolysis can also be converted to alanine by the action of ALT. Prediabetic rats drinking WTEA presented decreased testicular protein levels of ALT (0.78 ± 0.16-fold change vs control) relative to the control group and prediabetic rats drinking water (0.98 ± 0.11-fold change vs control) (Figure 5.3d). However, no differences were found in testicular alanine content among the experimental groups (Figure

5.3e). Lactate/alanine ratio was also determined as it reflects the NADH/NAD^+ equilibrium and thus the tissue redox state. A decreased testicular lactate/alanine ratio was observed in PreDM (2.4 ± 0.08) and PreDM+WTEA (2.6 ± 0.08) groups relative to the control (3.6 ± 0.15).

Lactate can be exported to the intratubular fluid by MCT4. Thus, we measured testicular protein levels of this transporter. Our results demonstrated that WTEA ingestion by prediabetic animals led to decreased testicular protein levels of MCT4 (0.87 ± 0.18-fold change vs control), compared to prediabetic rats drinking water (1.19 ± 0.19-fold change vs control). However, no changes were observed relative to the control group (Figure 5.3f). Moreover, after performing a correlation analysis, we detected a strong positive correlation between testicular lactate content and MCT4 protein levels (r = 0.9010; p = 0.0368) in the group of prediabetic animals drinking WTEA (Table 5.4). No other correlations were found in the testicular tissue.

Table 5.4 Relationships between lactate content, lactate dehydrogenase protein levels and activity, monocarboxylate transporter 4 protein levels, alanine content and alanine transaminase protein levels evaluated by Pearson's correlation coefficient (r) in testis from the control, prediabetic rats drinking water (PreDM) or white tea (PreDM+WTEA).

	Testis		
	CTRL	PreDM	PreDM+WTEA
Lactate content versus LDH protein levels	r = 0.5580 p = 0.2498	r = 0.7527 p = 0.0842	r = 0.7927 p = 0.1097
Lactate content versus LDH activity	r = -0.2486 p = 0.6347	r = -0.1170 p = 0.8254	r = 0.6613 p = 0.2242
Lactate content versus MCT4 protein levels	r = -0.0966 p = 0.8556	r = 0.3621 p = 0.4805	r = 0.9010 p = 0.0368*
Alanine content versus ALT protein levels	r = 0.4489 p = 0.3719	r = 0.6993 p = 0.1220	r = -0.6284 p = 0.1815

Abbreviations: MCT4, monocarboxylate transporter 4; LDH, lactate dehydrogenase; ALT, alanine transaminase. Differences with $p < 0.05$ were considered statistically significant.

Cauda epididymis metabolism

Prediabetes altered the protein levels of key glycolysis intervenient in epididymis which were maintained by white tea consumption

To evaluate cauda epididymis glycolytic profile, we measured GLUTs and PFK1 protein levels, as well as PFK1 activity. Our results demonstrated a decrease in GLUT1 protein levels in the epididymis of PreDM 0.81 ± 0.05-fold change vs control) and PreDM+WTEA rats (0.78 ± 0.05-fold change vs control) groups (Figure 5.4a). Epididymis GLUT2 protein levels also decreased (0.84 ± 0.09-fold change vs control) in prediabetic rats drinking water (Figure 5.4b). Notably, WTEA consumption by prediabetic rats increased epididymis GLUT2 protein levels (1.0 ± 0.16-fold change vs control) relative to PreDM group, showing a similar level relative to the control group. It was also observed a decrease in epididymis GLUT3 protein levels in the prediabetic animals drinking water (0.64 ± 0.13-fold change vs control) or WTEA (0.42 ± 0.04-fold change vs control) relative to the control group (Figure 5.4c). In addition, epididymis GLUT3 protein levels of PreDM+WTEA group decreased when compared with PreDM group. Concerning epididymis PFK1

protein levels, there was a decrease in PreDM group (0.85 ± 0.04-fold change vs control) relative to control. The ingestion of WTEA by prediabetic animals maintained epididymis PFK1 levels to normal levels (Figure 5.5a). Besides, there were no changes in PFK1 activity among the experimental groups (Figure 5.5b).

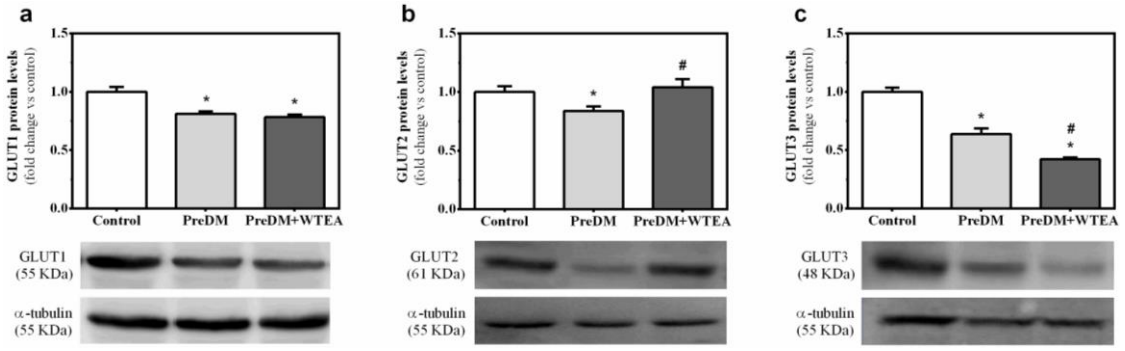


Figure 5.4 Effect of white tea (WTEA) consumption in epididymal glucose transport in prediabetic rats. The figure shows pooled data of independent experiments, indicating glucose transporters, GLUT1 (a), GLUT2 (b) and GLUT3 (c) protein levels in control, prediabetic rats drinking water (PreDM) or white tea (PreDM+WTEA). The representative blots (of one sample per each condition) of GLUT1, GLUT2 and GLUT3 are also shown. Variation in protein levels is presented as fold change vs control. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as relatively to: * control or # PreDM.

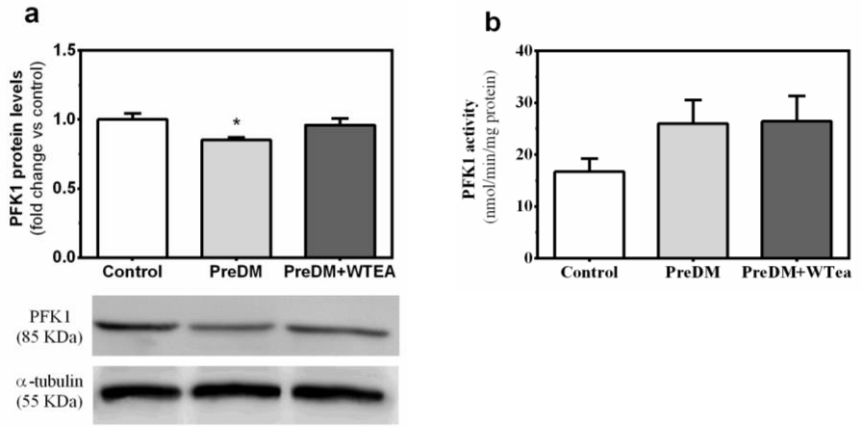


Figure 5.5 Effect of white tea (WTEA) consumption in epididymal glycolytic profile in prediabetic rats. The figure shows pooled data of independent experiments, indicating phosphofructokinase 1 (PFK1) protein levels (a) and activity (b) in control, prediabetic rats drinking water (PreDM) or white tea (PreDM+WTEA). The representative blots (of one sample per each condition) of PFK1 are also shown. Variation in protein levels is presented as fold change vs control. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as relatively to: * control.

Prediabetes-induced alterations in LDH protein levels and LDH activity were prevented by white tea ingestion

After evaluating epididymis LDH protein levels, we verified a decrease in PreDM group to 0.75 ± 0.04-fold change vs control (Figure 5.6a). In PreDM+WTEA group there was an increase in epididymis LDH protein levels to 1.1 ± 0.18-fold change vs control ($p = 0.0047$) relative to the PreDM group. On the other hand, LDH activity increased ($p = 0.014$) in PreDM group (110.1 ±

1.8 nmol/min/mg protein) relative to the control group (98.1 ± 3.4 nmol/min/mg protein) (Figure 5.6b). Prediabetic animals drinking WTEA did not show alterations in epididymal LDH activity (97.3 ± 6.0 nmol/min/mg protein) relative to the control group. Concerning epididymis lactate content, there was a decrease (p = 0.012) in the PreDM group (3760 ± 290 pmol/mg_{ww}) when compared to the control (5878 ± 610 pmol/mg_{ww}) (Figure 5.6c). In the group of prediabetic animals drinking WTEA, there was also a decrease (p = 0.025) in epididymal lactate content (3289 ± 651 pmol/mg_{ww}) relative to the control. Besides, a strong positive correlation was detected between epididymal lactate content and LDH activity (r = 0.9174; p = 0.0281) in the control group (Table 5.5). No other correlations were found in the epididymis.

Prediabetes induced a decrease in epididymis ALT and MCT4 protein levels as well as in alanine content while ingestion of white tea maintained their levels

It was observed a decrease in epididymis ALT protein levels of PreDM group (0.77 ± 0.07-fold change vs control; p = 0.016) relative to the control group, but not in PreDM+WTEA group (1.2 ± 0.22-fold change vs control; p = 0.0064) (Figure 5.6d). There was also a decrease in alanine content in the prediabetic animals drinking water (518 ± 55 pmol/mg_{ww}; p = 0.0083) when compared to the control group (760 ± 26 pmol/mg_{ww}) (Figure 5.6e). The ingestion of WTEA by prediabetic rats led to an epididymis alanine content of 484 ± 106 pmol/mg_{ww}, showing no differences relative to the control or PreDM groups. Notably, the lactate/alanine ratio was not altered in the epididymis of the different experimental groups. Concerning epididymis MCT4 protein levels, there was a decrease in PreDM group (0.76 ± 0.04-fold change vs control; p = 0.0028), while the consumption of WTEA by prediabetic rats maintained its levels in relation to the control group (1.11 ± 0.21-fold change vs control; p = 0.0085) (Figure 5.6f).

White tea ingestion by prediabetic rats improved sperm motility and maintained sperm viability to normal levels

To evaluate epididymis sperm quality, we have determined the following parameters: motility, viability, density and morphology (Table 5.6). The ingestion of WTEA by prediabetic rats led to an increase in sperm motility (77 ± 1%) relative to the control (65 ± 4%; p = 0.0125) and the group of prediabetic rats drinking water (71 ± 2%; p = 0.0278). However, sperm viability decreased (p = 0.0175) in PreDM group (24 ± 2%) when compared to the control group (33 ± 2%). The ingestion of WTEA maintained sperm viability to normal levels (29 ± 1%). No differences were found in sperm density of rats from the different experimental groups, which presented 3.1 ± 0.7, 4.7 ± 0.4 and 5.4 ± 0.9 cells x10^7/mL in the control, PreDM and PreDM+WTEA groups, respectively (Table 5.6). There was an increase in spermatozoa abnormal morphology in the groups of prediabetic animals drinking water (67 ± 4%; p = 0.0001) or WTEA (64 ± 5%; p = 0.0006) relative to the control (39 ± 1%).

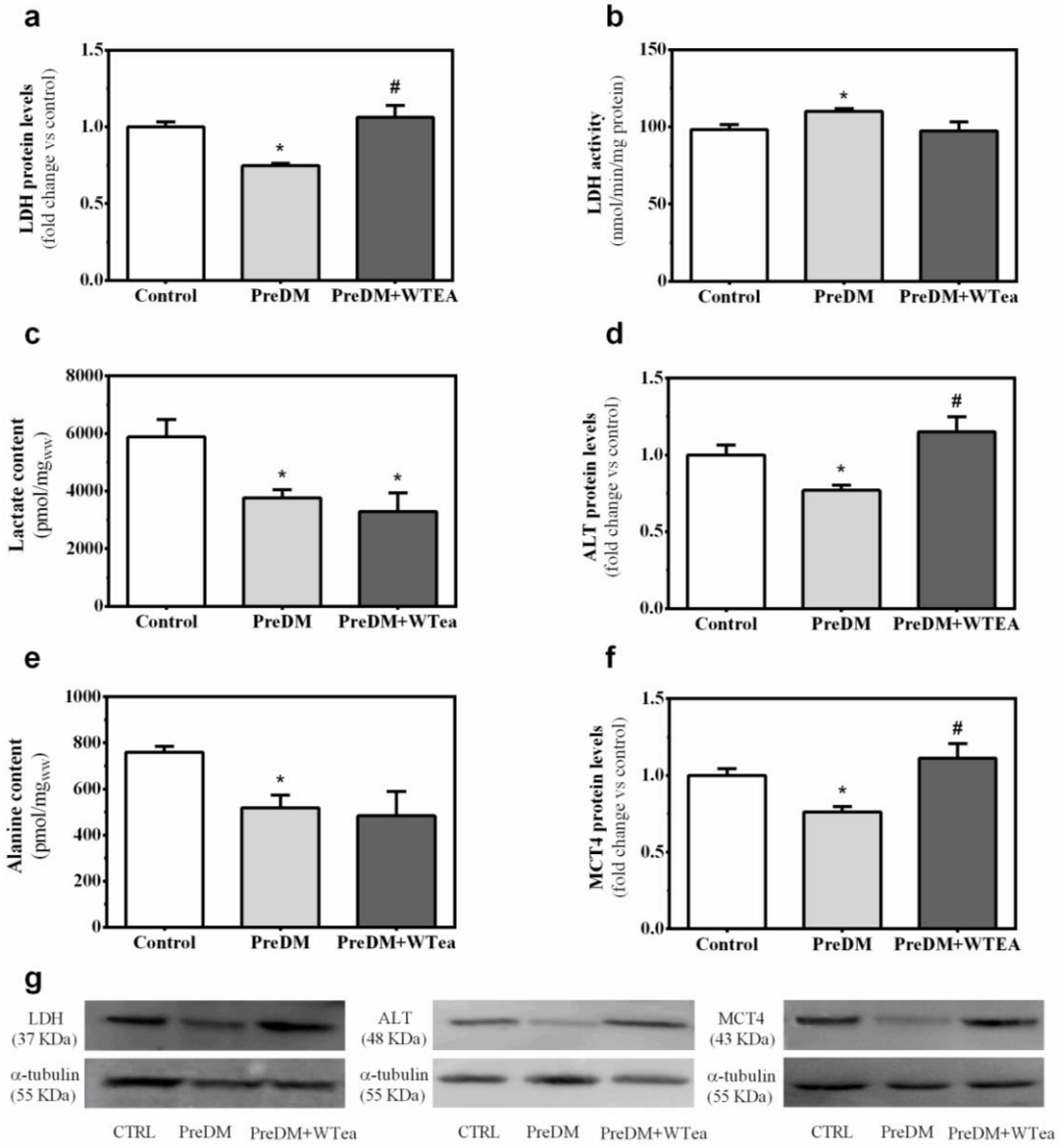


Figure 5.6 Effect of white tea (WTEA) consumption in the epididymal content of lactate and alanine in prediabetic rats. The figure shows pooled data of independent experiments, indicating lactate dehydrogenase (LDH) protein levels (a), LDH activity (b) and lactate content (c) in control, prediabetic rats drinking water (PreDM) or white tea (PreDM+WTEA). The figure also shows alanine transaminase (ALT) protein levels (d), alanine content (e) and monocarboxylate transporter 4 (MCT4) protein levels (f). The representative blots (of one sample per each condition) of LDH, ALT and MCT4 are also shown (g). Variation in protein levels is presented as fold change vs control. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as relatively to: * control or # PreDM.

Table 5.5 Relationships between lactate content, lactate dehydrogenase protein levels and activity, monocarboxylate transporter 4 protein levels, alanine content and alanine transaminase protein levels evaluated by Pearson's correlation coefficient (r) in epididymis from the control, prediabetic rats drinking water (PreDM) or white tea (PreDM+WTEA).

	Epididymis		
	CTRL	PreDM	PreDM+WTEA
Lactate content versus LDH protein levels	r = -0.2549 p = 0.6790	r = 0.1922 p = 0.7568	r = 0.3416 p = 0.5737
Lactate content versus LDH activity	r = 0.9174 p = 0.0281*	r= 0.1598 p = 0.7974	r = 0.4041 p = 0.4998
Lactate content versus MCT4 protein levels	r = -0.3394 p = 0.5763	r = 0.3458 p = 0.5686	r = 0.3040 p = 0.6189
Alanine content versus ALT protein levels	r = -0.4472 p = 0.4504	r = -0.1776 p = 0.7750	r = -0.4136 p = 0.4888

MCT4, monocarboxylate transporter 4; LDH, lactate dehydrogenase; ALT, Alanine transaminase. Differences with $p < 0.05$ were considered statistically significant.

Table 5.6 Effect of white tea consumption in epididymal sperm quality (motility, viability, density and morphology) in prediabetic rats.

Sperm Parameters	Control	PreDM	PreDM+WTEA
Motility (%)	65 ± 4	71 ± 2	77 ± 1 $^{*\#}$
Viability (%)	33 ± 2	24 ± 2 *	29 ± 1
Density (cells x 10^7/mL)	3.1 ± 0.7	4.7 ± 0.4	5.4 ± 0.9
Abnormal Morphology (%)	39 ± 1	67 ± 4 *	64 ± 5 *

Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are indicated as relatively to: * control. PreDM, prediabetic rats drinking water; PreDM+WTEA, prediabetic rats drinking white tea.

Discussion

Modern societies face a constant fight against DM because it is an incurable lifelong disease with spontaneous manifestation and an alarming prevalence worldwide [47]. It is of extreme relevance to study prediabetes to counteract the progression of DM. The diagnostic of prediabetes is a serious wake-up call, and its timely and adequate treatment may turn things around. As glucose homeostasis is compromised in individuals with prediabetes and glucose constitutes the main fuel to reproductive metabolic pathways, the impairment of glucose homeostasis seems to be on the basis of male subfertility/infertility evidenced in prediabetic individuals [48]. Male fertility depends on the successful development of spermatozoa in the testes, although testicular sperm are usually immotile. The acquisition of motility and the establishment of a mature swimming pattern are the most evident maturational changes occurring during spermatozoa transit throughout the epididymis [49]. Therefore, sperm quality can be indirectly influenced by alterations in testicular and epididymal physiology. It is crucial to control the metabolic alterations induced by prediabetes at testicular and epididymal levels to preserve sperm quality and avoid male reproductive problems.

Several antidiabetic drugs, including metformin and pioglitazone, are extensively used in the treatment of T2DM. These antidiabetics have shown ability to modulate testicular cells metabolism, influencing sperm quality [50, 51]. Still, pharmacological agents are often

associated with several undesired side effects [42]. To overcome these deleterious effects of conventional antidiabetic drugs, natural products have been considered for DM treatment [51]. For instance, WTEA has demonstrated hypoglycemic and antioxidant activities [51], being also a modulator of spermatogenesis [52]. Though most of WTEA potentialities are still poorly studied, it is known that its high catechin content confers it a higher antioxidant activity over other types of tea [11] and is associated with most of its health-promoting effects [19]. Testicular function is highly controlled by SCs and is very susceptible to high oxidative stress (OS) levels, even in normal physiological conditions, due to their high metabolic rates [29]. The addition of a WTEA extract to rat SCs culture medium induced metabolic alterations that may be important for male fertility preservation [29]. We have also verified that caffeine, one of WTEA's main components, induced an increased production of lactate and alanine in human SCs and altered their oxidative profile [33]. Moreover, the addition of a WTEA extract to spermatozoa storage medium strongly improved sperm viability [53]. Besides, i*n vivo* studies using an STZ-induced animal model of prediabetes, demonstrated that WTEA improves cardiac [54] and brain [55] metabolic and oxidative profiles. Most recently, we observed that WTEA daily ingestion by prediabetic rats improves sperm quality by decreasing testicular OS [56]. However, most of the metabolic pathways and mechanisms that may promote reproductive dysfunction in prediabetic individuals remain to be understood. Herein, we aimed to evaluate the effects of WTEA against testicular and epididymal metabolic alterations induced by prediabetes.

Our data showed that STZ-treated animals presented several characteristics related to the development of prediabetes. At the end of the treatment, they presented blood glucose levels within the prediabetic range (100-125 mg/dL) [54], glucose intolerance and insulin resistance. The daily ingestion of WTEA did not decrease blood glucose to normal levels, but rather improved glucose tolerance and insulin sensitivity. This is in accordance with previous studies reporting the potential action of catechins and their derivatives to improve glucose intolerance [54, 57] and insulin sensitivity [58]. Previous evidence suggested that these effects are mostly due to the potent action of catechins and their ability to scavenge reactive oxygen species (ROS), improving the cellular OS [54].

Testicular and epididymal cells, which are irrigated by the testicular artery, uptake glucose from the bloodstream by a facilitated diffusion mechanism mediated by GLUTs. These transporters regulate the bidirectional movement of glucose between the extracellular and intracellular compartments, with passive diffusion down its concentration gradient, maintaining a constant supply of glucose available for cell metabolism [58]. The development of prediabetes induced an increase in GLUT3 protein levels, but not in GLUT1 nor GLUT2, in the testes. Previous studies using animal models of prediabetes (induced by a high-energy diet) and T2DM also reported increased testicular protein levels of GLUT3 [59] and non-altered protein levels of GLUT2 [60], respectively. As glucose levels are higher than normal in the plasma of prediabetic animals, the difference between extracellular and intracellular glucose

concentration is higher. Thus, more glucose is being directed to the intracellular compartment due to the concentration gradient, leading to upregulation of GLUTs. We have to consider that the kinetic properties differ between the several GLUT isoforms [61]. GLUT3 has a higher affinity for glucose than GLUT1, ensuring a maximal glucose uptake in normal physiological conditions. This explains why GLUT3, but not GLUT1, was increased in PreDM group. GLUT1 plays a constitutive role, as it is responsible for the basal glucose uptake required to maintain respiration [62]. Contrarily, while GLUT2 has high capacity for glucose transport, it has very low affinity, thus being very sensitive to glucose fluctuations [63]. When circulating glucose levels are high, this hexose is transported to the intracellular compartment through GLUT2 [54]. Thus, the increase in GLUT2 levels in PreDM+WTEA animals could be due to the detected slight hyperglycemia. However, GLUT2 protein levels were not altered in prediabetic rats drinking water, which is in accordance with previous studies that also noted that GLUT2 is not altered in testicular cells of prediabetic rats [57, 64]. As GLUT2 is not insulin-dependent, GLUT2 protein levels may not be altered due to impairment of glucose tolerance in testicular tissue of prediabetic animals. Though, the flux of glucose through GLUT2 may be impaired. Further studies will be needed to evaluate the transport rates of this transporter in testicular cells of prediabetic rats. Prediabetic rats drinking WTEA also showed increased GLUT3 protein levels, which is in accordance with the persistent mild hyperglycemia evidenced in this group. Additionally, WTEA led to increased GLUT2 protein levels relative to the control but not to PreDM group. This upregulation of GLUT2 may be due to higher glycemic levels evidenced in this group relative to control. These results agree with our previous suggestion that glucose intolerance influences glucose uptake by GLUT2. The improvement of glucose tolerance by WTEA ingestion triggered a normal response of GLUT2 to the higher blood glucose levels evidenced in PreDM+WTEA group.

While the precise demand for metabolic substrates within the epididymis are unknown, a supply of energy is required for the maintenance of cellular processes. Interestingly, there is a greater rate of glucose metabolism in the testes than in the epididymides [54]. In fact, our results reflect a different profile in glucose uptake in cauda epididymis relative to the testis. The prediabetic condition led to decreased epididymal protein levels of GLUT1, GLUT2 and GLUT3, indicating a different response to the impairment of glucose homeostasis in prediabetic animals and tissue-specific metabolic needs. As prediabetic animals presented mild hyperglycemia, the flux of glucose is being directed to the intracellular compartment. However, downregulation of GLUTs may reflect an adaptation of cauda epididymis to avoid an excessive entrance of glucose, as previously reported [54]. The ingestion of WTEA maintained GLUT2 to normal levels, but further decreased GLUT3 protein levels, illustrating a sensitiveness of GLUT2 to the improvement of glucose tolerance. The decrease in GLUT3 was also observed in other tissues of prediabetic rats, such cerebral cortex [65] and intestine [66]. This downregulation was suggested to be due to a competitive inhibiting action of tea polyphenols with this transporter, as already reported [67].

After glucose uptake, it is metabolized through glycolysis, where PFK1 has a major role. The glycolysis end-product, pyruvate, can be imported to the mitochondria to fuel the Krebs cycle or it can be converted to lactate by LDH and then exported to the extracellular medium [68]. Testicular PFK1 and LDH protein levels, as well as PFK1 activity, were not altered by prediabetes. However, LDH activity was decreased in PreDM group, resulting in a lower testicular lactate content. A reduction in testicular lactate content was also observed in a STZ-induced T2DM animal model [69], indicating that both prediabetes and T2DM induce similar damages. As PFK1 protein levels and activity were not altered by the prediabetic condition, a normal production of pyruvate seems to be occurring. Thus, the reduced lactate content is surely correlated with the decrease in LDH activity in the testicular tissue of PreDM rats. Our data suggests that prediabetes alters the testicular metabolic profile by inhibiting its preferred testicular energetic pathway and compromising lactate accumulation.

The ingestion of WTEA by prediabetic rats decreased testicular PFK1 protein levels, while stimulating its activity (Figure 5.2b). A previous study, in rat SCs, also reported a decrease in PFK1 levels after exposure to a WTEA extract, although no mention was made regarding the impact on the activity of this enzyme [70]. SCs present a high glycolytic flux and although PFK1 levels were decreased, lactate production by these cells was not compromised [69]. The same pattern was observed in the present study. Although LDH activity was decreased in PreDM+WTEA group to lower levels than in the group of prediabetic rats drinking water, testicular lactate content was sustained. It is known that the conversion of pyruvate to lactate depends on the stoichiometric pressure on each side of the equation [70]. Thus, the increase of pyruvate production due to the augmented PFK1 activity potentiates the conversion of pyruvate to lactate, counteracting the decrease in LDH activity. We hypothesize that the increase in PFK1 activity induced by WTEA was triggered by the increase in glucose uptake, as observed by GLUTs upregulation, and contributed to the restoration of lactate production to normal levels. Overall, these results suggest that WTEA triggers an adaptive response of testicular tissue to ensure the correct lactate production, which is essential for germ cell survival and development [70]. The activity of LDH is regulated by various factors [71]. For instance, alterations in glucose uptake, as noted in prediabetic rats, may have an impact in LDH activity. Our results suggest that WTEA ingestion may be a good approach to maintain a normal testicular lactate content in prediabetic animals, by enhancing glucose conversion to pyruvate, keeping the preferred testicular metabolic pathway functioning.

Interestingly, a distinct pattern was observed in the metabolic profile of cauda epididymis. Prediabetes reduced PFK1 protein levels but did not alter PFK1 activity. Moreover, LDH protein levels were decreased by the prediabetic condition and despite the increase in LDH activity, lactate content in cauda epididymis was reduced. Cauda epididymis metabolism is reported to rely on glycolysis and mitochondrial phosphorylation for ATP production [72], involving or being limited by pyruvate levels [73]. In fact, we observed that PFK1 enzyme is 3-fold more active in epididymis (Figure 5.5b) than in testis (Figure 5.2b). As epididymal glucose uptake was strongly

reduced in prediabetic rats and there was also a decrease in PFK1 protein levels, pyruvate production could be reduced, as well as ATP production. WTEA ingestion by prediabetic rats maintained PFK1 protein levels, as well as LDH protein levels and activity, although, lactate content remained lower than in control group. Lactate accumulation in cauda epididymis is inhibited under prediabetic conditions, thus favoring an increased state of oxidative phosphorylation [74]. Notably, WTEA ingestion could maintain the epididymal energy production. In control conditions, there was a positive correlation between lactate production and LDH activity, which was not found in prediabetic rats drinking water or WTEA, indicating a crucial metabolic dysfunction induced by the disease. Despite the favorable effects on the energetic metabolism of epididymal tissue, WTEA ingestion did not change the correlation observed in the control.

Furthermore, besides being converted to lactate, pyruvate can also be used to produce alanine by a reversible reaction catalyzed by ALT [75]. In testis, ALT protein levels were decreased in prediabetic rats drinking WTEA (PreDM+WTEA) comparatively to prediabetic rats drinking water (PreDM) and to control group. However, testicular alanine levels were not altered by prediabetes itself nor WTEA consumption by those rats. Both pyruvate and lactate can be exported to the extracellular compartment through MCT4 [76]. The protein levels of this transporter in testis were not altered by the prediabetic condition, however, there was an adjustment induced by WTEA. These results suggest an improvement in lactate efflux to germ cells in prediabetic rats induced by WTEA consumption, which was proven by the positive correlation found between lactate production and MCT4 protein levels exclusively in the PreDM+WTEA group.

Regarding the epididymal tissue, the decrease in lactate content in PreDM group was accompanied by a decrease in ALT protein levels, leading to a diminished epididymal alanine content. Moreover, MCT4 protein levels were also reduced. These results support our previous suggestion that epididymal metabolism of prediabetic animals may be directed to mitochondria. Once pyruvate is not being preferentially used to produce lactate or alanine and the export of lactate and pyruvate to the extracellular compartment by MCT4 is inhibited, it strongly suggests that the majority of pyruvate is being used for Krebs cycle. The ingestion of WTEA by prediabetic rats maintained epididymal ALT and MCT4 protein levels, as well as alanine content similar to the control group. Overall, the ingestion of WTEA by prediabetic rats maintained almost all the control points of epididymal metabolism under normal values, except for lactate production.

The maintenance of spermatozoa functionality is crucial for male fertility preservation. Thus, we also evaluated the effect of WTEA ingestion by prediabetic rats in the quality of epididymal spermatozoa, including viability, morphology, motility and density. Our results showed that prediabetes induced a decrease in sperm viability relative to the control. This may be a result of the diminished testicular and epididymal lactate content observed in this group, particularly

in testis, as lactate exerts an anti-apoptotic activity in developing germ cells [77]. Moreover, a higher percentage of abnormal spermatozoa was also observed in PreDM group. Spermatozoa abnormalities are usually associated with increased OS, resulting from the metabolic changes induced by prediabetes [78]. It has been reported that the redox state of tissues can be estimated by measuring the lactate/alanine ratio, which reflects the NADH/NAD^+ ratio [79]. In fact, we have observed a decrease in testicular lactate/alanine ratio in the PreDM group (data not shown), indicating that prediabetes altered the testicular redox state, which might be associated with the observed increase in spermatozoa abnormalities. Concerning the group of prediabetic rats drinking WTEA, there was an increase in sperm motility relative to control and PreDM groups. It has been reported that increased ROS production leads to decreased motility [80]. Thus, we believe that the potent antioxidant activity of WTEA was able to diminish ROS levels and improve sperm motility. Moreover, the improvement of testicular lactate content and epididymal pyruvate production in PreDM+WTEA group relative to the PreDM may also be responsible for the observed improvement in motility. In fact, pyruvate and lactate are very efficient in supporting the oxidative metabolism of bovine epididymal spermatozoa [81]. Interestingly, spermatozoa are the unique cellular type that can use lactate directly for mitochondrial respiration because they have an LDH-specific isoform [80]. The ingestion of WTEA by prediabetic rats maintained sperm viability to normal levels. As lactate is an anti-apoptotic factor to germ cells, and testicular lactate content was normal in prediabetic rats drinking WTEA, it may explain why sperm viability was also normal. Nevertheless, animals from the PreDM+WTEA did not show a normal testicular lactate/alanine ratio, thus, the epididymal spermatozoa from this experimental group also presented a high percentage of abnormal sperm, owing to the altered testicular redox state induced by the prediabetic condition [82].

In conclusion, our results indicate that although overlooked, epididymal metabolism is as important as testicular metabolism for sperm quality and hence male fertility preservation. The ingestion of WTEA by prediabetic rats allowed the adjustment of testicular metabolism to maintain lactate and alanine content under normal levels, preserving germ cell energetic supplies. Regarding epididymal metabolism, the production of lactate is neglected to maintain glycolytic substrates that are crucial for spermatozoa maturation and motility acquisition. Our results reinforce the importance of glucose tolerance improvement to male fertility in the face of prediabetes. Daily ingestion of WTEA may be an inexpensive and feasible strategy to counteract the metabolic dysfunctions induced by the prediabetes.

References

1. Slaughter GR and Means AR (1983) Follicle-stimulating hormone activation of glycogen phosphorylase in the Sertoli cell-enriched rat testis. Endocrinology 113(4):1476-85.
2. Russell LD (1993) Form, dimensions, and cytology of mammalian Sertoli cells. In: LD Russell and MD Griswold (eds) The Sertoli Cell, Cache River Press, Clearwater pp. 1–37
3. Xiong WP, et al. (2009) Apoptotic spermatogenic cells can be energy sources for Sertoli cells. Reproduction 137(3):469-79.
4. Alves MG, et al. (2013) Diabetes, insulin-mediated glucose metabolism and Sertoli/blood-testis barrier function. Tissue Barriers 1(2):e23992.

5. Spiro MJ (1984) Effect of diabetes on the sugar nucleotides in several tissues of the rat. Diabetologia 26(1):70-5.
6. Walker W and Cheng J (2005) FSH and testosterone signaling in Sertoli cells. Reproduction 130(1):15-28.
7. Cheng CY, et al. (2010) Regulation of spermatogenesis in the microenvironment of the seminiferous epithelium: new insights and advances. Mol Cell Endocrinol 315(1-2):49-56.
8. Russell LD, et al. (1990) A comparative study in twelve mammalian species of volume densities, volumes, and numerical densities of selected testis components, emphasizing those related to the Sertoli cell. American Journal of Anatomy 188(1):21-30.
9. Sharpe RM, et al. (2003) Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction 125(6):769-84.
10. Petersen C and Soder O (2006) The Sertoli cell-a hormonal target and'super'nurse for germ cells that determines testicular size. Hormone Research 66(4):153-61.
11. Rato L, et al. (2010) Tubular fluid secretion in the seminiferous epithelium: ion transporters and aquaporins in Sertoli cells. Journal of Membrane Biology 236(2):215-24.
12. Koskimies A and Kormano M (1973) The proteins in fluids from the seminiferous tubules and rete testis of the rat. Reproduction 34(3):433-34.
13. Fisher D (2002) New light shed on fluid formation in the seminiferous tubules of the rat. J Physiol 542(Pt 2):445-52.
14. Setchell BP (1970) The secretion of fluid by the testes of rats, rams and goats with some observations on the effect of age, cryptorchidism and hypophysectomy. J Reprod Fertil 23(1):79-85.
15. Tuck R, et al. (1970) The composition of fluid collected by micropuncture and catheterization from the seminiferous tubules and rete testis of rats. Pflügers Archiv European Journal of Physiology 318(3):225-43.
16. Jenkins AD, et al. (1980) Concentrations of seven elements in the intraluminal fluids of the rat seminiferous tubules, rate testis, and epididymis. Biol Reprod 23(5):981-7.
17. Clulow J and Jones R (2004) Composition of luminal fluid secreted by the seminiferous tubules and after reabsorption by the extratesticular ducts of the Japanese quail, Coturnix coturnix japonica. Biology of Reproduction 71(5):1508.
18. Oliveira PF, et al. (2009) Intracellular pH regulation in human Sertoli cells: role of membrane transporters. Reproduction 137(2):353-59.
19. Oliveira PF, et al. (2009) Membrane Transporters and Cytoplasmatic pH Regulation on Bovine Sertoli Cells. Journal of Membrane Biology 227(1):49-55.
20. Roos A and Boron WF (1981) Intracellular pH. Physiol Rev 61(2):296-434.
21. Boron W (2004) Regulation of intracellular pH. Advances in Physiology Education 28(4):160-79.
22. Jegou B, et al. (1982) Seminiferous tubule fluid and interstitial fluid production. I. Effects of age and hormonal regulation in immature rats. Biology of Reproduction 27(3):590-95.
23. Fernandez MF, et al. (2012) Semen quality and reproductive hormone levels in men from Southern Spain. International Journal of Andrology 35(1):1-10.
24. Jorgensen N, et al. (2001) Regional differences in semen quality in Europe. Human Reproduction 16(5):1012-19.
25. Jorgensen N, et al. (2002) East-West gradient in semen quality in the Nordic-Baltic area: a study of men from the general population in Denmark, Norway, Estonia and Finland. Human Reproduction 17(8):2199-208.
26. Nordkap L, et al. (2012) Regional differences and temporal trends in male reproductive health disorders: semen quality may be a sensitive marker of environmental exposures. Molecular and cellular endocrinology 355(2):221-30.
27. Bustos-Obregón E and Hartley B (2008) Ecotoxicology and Testicular Damage (Environmental Chemical Pollution): A Review. International Journal of Morphology 26(4):833-40.
28. Mathur PP and D'Cruz SC (2011) The effect of environmental contaminants on testicular function. Asian J Androl 13(4):585-91.
29. Sharpe RM (2010) Environmental/lifestyle effects on spermatogenesis. Philos Trans R Soc Lond B Biol Sci 365(1546):1697-712.
30. Goulis DG and Tarlatzis BC (2008) Metabolic syndrome and reproduction: I. testicular function. Gynecological Endocrinology 24(1):33-39.
31. Mah PM and Wittert GA (2010) Obesity and testicular function. Molecular and Cellular Endocrinology 316(2):180-86.
32. Bonde JP and Storgaard L (2002) How work place conditions, environmental toxicants and lifestyle affect male reproductive function. International Journal of Andrology 25(5):262-68.
33. Suehiro RM, et al. (2008) Testicular Sertoli cell function in male systemic lupus erythematosus. Rheumatology 47(11):1692-97.
34. Karagiannis A and Harsoulis F (2005) Gonadal dysfunction in systemic diseases. European Journal of Endocrinology 152(4):501-13.
35. Sartorius GA and Handelsman DJ (2010) Testicular Dysfunction in Systemic Diseases. In: E Nieschlag, HM Behre and S Nieschlag (eds) Andrology: Male Reproductive Health and Dysfunction., Springer Berlin pp. 339-64

36. Setchell B (1980) The Functional Significance of the Blood-testis Barrier. Journal of andrology 1(1):3-10.
37. Su L, et al. (2011) Drug transporters, the blood-testis barrier, and spermatogenesis. J Endocrinol 208(3):207-23.
38. Wong CH and Cheng CY (2005) The blood-testis barrier: its biology, regulation, and physiological role in spermatogenesis. Curr Top Dev Biol 71:263-96.
39. Toyama Y, et al. (2003) Ectoplasmic specializations in the Sertoli cell: new vistas based on genetic defects and testicular toxicology. Anat Sci Int 78(1):1-16.
40. Mazaud-Guittot S, et al. (2010) Claudin 11 deficiency in mice results in loss of the Sertoli cell epithelial phenotype in the testis. Biol Reprod 82(1):202-13.
41. Lui WY and Cheng CY (2007) Regulation of cell junction dynamics by cytokines in the testis: a molecular and biochemical perspective. Cytokine Growth Factor Rev 18(3-4):299-311.
42. Cheng CY and Mruk DD (2009) An intracellular trafficking pathway in the seminiferous epithelium regulating spermatogenesis: a biochemical and molecular perspective. Crit Rev Biochem Mol Biol 44(5):245-63.
43. Waites G and Gladwell R (1982) Physiological significance of fluid secretion in the testis and blood-testis barrier. Physiol Rev 62(2):624-71.
44. Russell LD (1978) The blood-testis barrier and its formation relative to spermatocyte maturation in the adult rat: a lanthanum tracer study. Anat Rec 190(1):99-111.
45. Siu MKY and Cheng CY (2009) Extracellular matrix and its role in spermatogenesis. In: CY Cheng (ed) Molecular Mechanisms in Spermatogenesis, Landes Bioscience, Austin pp. 74-91
46. Setchell BP (1986) The movement of fluids and substances in the testis. Australian Journal of Biological Sciences 39(2):193-207.
47. Setchell BP (2009) Blood-testis barrier, junctional and transport proteins and spermatogenesis. Advances in Experimental Medicine and Biology 636:212-33.
48. Gaemers IC, et al. (1998) Differential expression pattern of retinoid X receptors in adult murine testicular cells implies varying roles for these receptors in spermatogenesis. Biology of Reproduction 58(6):1351-6.
49. Hogarth CA and Griswold MD (2010) The key role of vitamin A in spermatogenesis. Journal of Clinical Investigation 120(4):956-62.
50. Sugimoto R, et al. (2011) Retinoic acid metabolism links the periodical differentiation of germ cells with the cycle of Sertoli cells in mouse seminiferous epithelium. Mechanisms of Development 128(11-12):610-24.
51. Hess R and de Franca L (2009) Spermatogenesis and cycle of the seminiferous epithelium. In: CY Cheng (ed) Molecular Mechanisms in Spermatogenesis, Landes Bioscience/Springer Science, Austin pp. 1–15
52. Griswold M and McLean D (2006) The Sertoli cell. In: J Neill (ed) Knobil and Neill's physiology of reproduction, Elsevier, San Diego pp. 949-75
53. Aly HA, et al. (2010) Bacterial lipopolysaccharide-induced oxidative stress in adult rat Sertoli cells in vitro. Toxicology In Vitro 24(4):1266-72.
54. Bajpai M, et al. (1998) Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat. European Journal of Endocrinology 138(3):322-27.
55. Setchell BP (2004) Hormones: what the testis really sees. Reprod Fertil Dev 16(5):535-45.
56. Wenger RH and Katschinski DM (2005) The hypoxic testis and post-meiotic expression of PAS domain proteins. Seminars in Cell and Developmental Biology 16:547-53.
57. Gómez M, et al. (2009) Switches in 6-phosphofructo-2-kinase isoenzyme expression during rat sperm maturation. Biochemical and Biophysical Research Communications 387(2):330-35.
58. Boussouar F and Benahmed M (2004) Lactate and energy metabolism in male germ cells. Trends in Endocrinology and Metabolism 15(7):345-50.
59. Courtens JL and Ploen L (1999) Improvement of spermatogenesis in adult cryptorchid rat testis by intratesticular infusion of lactate. Biol Reprod 61(1):154-61.
60. Jutte N, et al. (1981) Exogenous lactate is essential for metabolic activities in isolated rat spermatocytes and spermatids. Reproduction 62(2):399-405.
61. Erkkila K, et al. (2002) Lactate inhibits germ cell apoptosis in the human testis. Molecular Human Reproduction 8(2):109-17.
62. Nakamura M, et al. (1982) Regulation of glucose metabolism by adenine nucleotides in round spermatids from rat testes. Journal of Biological Chemistry 257(23):13945-50.
63. Yanez AJ, et al. (2007) Expression of key substrate cycle enzymes in rat spermatogenic cells: fructose 1,6 bisphosphatase and 6 phosphofructose 1-kinase. Journal of Cellular Physiology 212(3):807-16.
64. Dias TR, et al. (2014) Sperm glucose transport and metabolism in diabetic individuals. Mol Cell Endocrinol 396(1-2):37-45.
65. Beckman J and Coniglio J (1979) A comparative study of the lipid composition of isolated rat Sertoli and germinal cells. Lipids 14(3):262-67.
66. Lynch KM, Jr. and Scott WW (1951) Lipid distribution in the Sertoli cell and Leydig cell of the rat testis as related to experimental alterations of the pituitary-gonad system. Endocrinology 49(1):8-14.

67. Retterstøl K, et al. (2001b) Metabolism of very long chain polyunsaturated fatty acids in isolated rat germ cells. Lipids 36(6):601-06.
68. Retterstol K, et al. (2001a) Studies on the metabolism of essential fatty acids in isolated human testicular cells. Reproduction 121(6):881-87.
69. Grootegoed J, et al. (1986) Metabolism of radiolabelled energy-yielding substrates by rat Sertoli cells. Reproduction 77(1):109.
70. Robinson R and Fritz I (1981) Metabolism of glucose by Sertoli cells in culture. Biol Reprod 24(5):1032-41.
71. Angulo C, et al. (1998) Hexose transporter expression and function in mammalian spermatozoa: cellular localization and transport of hexoses and vitamin C. Journal of Cellular Biochemistry 71(2):189-203.
72. Carosa E, et al. (2005) Ontogenetic profile and thyroid hormone regulation of type-1 and type-8 glucose transporters in rat Sertoli cells. Int J Androl 28(2):99-106.
73. Galardo M, et al. (2008) Regulation of expression of Sertoli cell glucose transporters 1 and 3 by FSH, IL1 , and bFGF at two different time-points in pubertal development. Cell and Tissue Research 334(2):295-304.
74. Ulisse S, et al. (1992) Thyroid hormone stimulates glucose transport and GLUT1 mRNA in rat Sertoli cells. Molecular and Cellular Endocrinology 87(1-3):131-37.
75. Kokk K, et al. (2004) Immunohistochemical detection of glucose transporters class I subfamily in the mouse, rat and human testis. Medicina (Kaunas) 40(2):156-60.
76. Piroli GG, et al. (2002) Peripheral glucose administration stimulates the translocation of GLUT8 glucose transporter to the endoplasmic reticulum in the rat hippocampus. Journal of Comparative Neurology 452(2):103-14.
77. Reagan LP, et al. (2001) Localization and regulation of GLUTx1 glucose transporter in the hippocampus of streptozotocin diabetic rats. Proceedings of the National Academy of Sciences of the United States of America 98(5):2820-5.
78. Rato L, et al. (2012) Metabolic Modulation Induced by Estradiol and DHT in Immature Rat Sertoli Cells cultured In Vitro. Biosci Rep 32(1):61-69.
79. Riera MF, et al. (2009) Molecular Mechanisms Involved in Sertoli Cell Adaptation to Glucose Deprivation. American Journal of Physiology Endocrinology and Metabolism 297(4):907-14.
80. Galardo MN, et al. (2007) The AMP-activated protein kinase activator, 5-aminoimidazole-4-carboxamide-1-b-D-ribonucleoside, regulates lactate production in rat Sertoli cells. J Mol Endocrinol 39(4):279-88.
81. Tosca L, et al. (2008) [AMPK: a link between metabolism and reproduction?]. Médecine Sciences (Paris) 24(3):297-300.
82. Galardo MN, et al. (2010) Adenosine regulates Sertoli cell function by activating AMPK. Mol Cell Endocrinol 330(1-2):49-58.

Chapter 6

General Discussion and Conclusions

Diabetes *mellitus* (DM) is one of the most prominent public health threats in modern societies and its prevalence is drastically increasing over the years [1, 2]. This metabolic disorder is mainly characterized by chronic hyperglycemia. Individuals with DM present disturbances of carbohydrate, fat and protein metabolism, which may result from defects in insulin secretion and/or insulin action [3]. Of particular importance is the key role of insulin in the regulation of glucose homeostasis in the body [4]. The dysregulation of glucose metabolism affects many glucose-dependent biological processes, including male reproductive function. For instance, a few hours of insulin deprivation in testicular cells can alter the nutritional support of spermatogenesis [5], which is the main process ensuring male fertility. Besides, DM is also associated with an overproduction of reactive oxygen species (ROS), which if uncontrolled can lead to oxidative stress (OS). OS is considered as one of the main factors for the increasing incidence of male subfertility/infertility. In fact, the fertilizing ability of young men seems to be decreasing over the last decades [6, 7].

Inadequate dietary patterns and a sedentary lifestyle play a key role in the current global epidemics of DM, as they promote an oxidative environment. It is of extreme relevance to reverse these lifestyle trends to decrease morbidity and mortality caused by metabolic diseases, but also to avoid its associated deleterious effects on male fertility. Many studies showed that antioxidant compounds obtained through the diet may have the potential to strengthen the reproductive tract antioxidant defense system and prevent fertility dysfunctions induced by DM. Tea is one of the most widely consumed beverages in the world, next to water [8, 9] and it is very rich in antioxidant compounds. In recent years, white tea (WTEA) aroused great interest due to its high antioxidant potential and associated health benefits. However, its role on male reproductive function has never been investigated.

In this project, we decided to use a WTEA from organic agriculture available in the Portuguese market. The phytochemical characterization of this WTEA was made by proton nuclear magnetic resonance (^{1}H-NMR) and compared to a green tea (GTEA) from the same brand and origin. There are controversial reports on which type of tea has the highest antioxidant potential, especially between WTEA and GTEA. Tea antioxidant potential is determined by its chemical composition, especially by its total catechins content. However, tea's chemical composition is influenced by many factors, including agricultural conditions, climate, leaves processing and origin, as well as the used experimental technique. Our selected WTEA showed about twice the total catechins content relative to GTEA, supporting its higher antioxidant power [10]. The most abundant compound in WTEA was the epigallocatechin gallate (EGCG), to which are ascribed many of the beneficial effects of tea. The second most abundant compound was caffeine, a methylxanthine that owes it popularity to its stimulant properties. WTEA was also very rich in L-theanine, a free amino acid almost exclusively found in *Camellia sinensis* leaves that is responsible for the relaxing properties of tea. EGCG, caffeine and L-theanine were considered the main bioactive WTEA components, as they represent the most

abundant compound of its phytochemical class: phenolic compounds, methylxanthines and free amino acids, respectively.

Since there were no studies regarding the effect of WTEA on male reproductive function, we firstly used an *in vitro* model of rat cultured Sertoli cells (SCs) obtained to evaluate the impact of a WTEA extract on SCs function. SCs are crucial for the maintenance of spermatogenesis as they provide the nutritional and physical support to the developing germ cells. Despite the limitations of the *in vitro-in vivo* data extrapolations concerning metabolic studies, an *in vitro* model of SCs is advantageous for reproductive toxicological studies [11]. Besides, the use of a tea extract allows the study of a higher concentration of phytochemicals relative to a tea infusion, allowing us to infer about its reproductive safety and possible medicinal use. Our first study showed that the selected WTEA extract (0.5 mg/mL) could modulate glucose metabolism by rat SCs, resulting in an increased lactate production [12]. As lactate is used as metabolic substrate and has an anti-apoptotic effect in the developing germ cells, the supplementation of SCs with WTEA extract may be advantageous to improve male reproductive health. Subsequently, to evaluate if there was a component of WTEA responsible for the outcome observed with the WTEA extract, we evaluated the individual effect of caffeine, EGCG and L-theanine on human SCs (hSCs) function. The use of hSCs allowed a better comparison with the *in vivo* features of these cells relative to rat SCs. Like the WTEA extract, caffeine at lowest concentrations (5 and 50 μM) altered hSCs glucose metabolism, resulting in the stimulation of lactate production [13]. On the other hand, the highest concentrations of caffeine (500 μM) [13], EGCG (50 μM) [14] and L-theanine (50 μM) were able to regulate hSCs metabolism and keep a normal lactate production. Based on the importance of lactate for spermatogenesis, these results indicate that both the WTEA extract and each compound individually at the chosen concentrations may be important for the regulation of hSCs function in specific conditions, and hence for male fertility. However, the highest concentration of caffeine (500 μM) induced a pro-oxidant potential in hSCs with a concurrent increase of protein oxidative damages [13]. This led us to conclude that a moderate consumption of caffeine (5-50 μM) appears to be safe for male reproductive health, whereas a heavy caffeine consumption (500 μM) may lead to deleterious effects in hSCs function and compromise spermatogenesis. Contrastingly, EGCG (50 μM) showed a protective role against oxidative damages to proteins and lipids, which may be very important to control the ROS overproduction induced by the high metabolic rates of hSCs [14]. Furthermore, while EGCG (50 μM) induced a decrease in hSCs proliferation and mitochondrial membrane potential, L-theanine (50 μM) had the opposite effect on these parameters. Overall, the analysis of each component individually allowed us to realize their distinct actions on hSCs function. Moreover, their action seems to complement each other when combined in the WTEA extract, showing better global results for SCs function when acting together.

SCs high metabolic rates are associated with high ROS levels [15], which may result in poor sperm quality [16]. Spermatozoa are highly susceptible to ROS attack due to their limited

intrinsic antioxidant capacity and lipidic membranes [17]. Sperm viability is one of the most important parameters of sperm quality and one of the most affected by ROS. Many researchers have been searching for new approaches to store spermatozoa in a viable state, mainly using refrigeration techniques such as cryopreservation. This is important not only to collect sperm from certain species under unexpected conditions, but also for its use in assisted reproductive technology (ART) [18]. However, sperm viability rapidly declines after storage in refrigerated environments due to the increase in ROS production over time [19]. Thus, it has been proposed that room temperature (RT) storage for short-term periods can be an effective alternative [20]. Various media have been tested to improve spermatozoa survival at RT [19] but viability is still abruptly decreasing in a short-time [19]. We decided to explore the possible protective effect of a WTEA extract in the survival and quality of rat epididymal spermatozoa. As spermatozoa are stored in cauda epididymis in a viable state until ejaculation, epididymal spermatozoa are often used in ART in humans and animals. This eliminates the variability caused by the post-translational modifications occurring during their transit through the male reproductive tract. The supplementation of a common sperm storage medium with WTEA extract (1 mg/mL) showed the best results in improving spermatozoa short-term survival at RT [10]. The addition of a WTEA extract was able to increase the antioxidant potential of the medium, resulting in the increase of spermatozoa antioxidant capacity. This is crucial to avoid deleterious effects of ROS accumulation over time, as reflected by the decrease in lipid peroxidation relative to the spermatozoa that were not incubated with tea extracts. Consequently, sperm viability was maintained as high as at collection time (0 h) during the 3 days of RT-storage [10]. WTEA extract can be an effective and affordable additive to spermatozoa storage medium to increase sperm survival rate and quality. This can be particularly important during transport of samples without refrigeration. Nevertheless, we wanted to explore the role of the most bioactive WTEA components, caffeine, EGCG and L-theanine, individually or in combination to the observed effect with the WTEA extract. We observed that the combination of the three compounds maintains a higher sperm viability over time, when compared to each compound individually. However, this mixture induced an increase in the oxidation of spermatozoa proteins relative to caffeine, EGCG or L-theanine separately [21]. Again, these results support the different action of these tea components when combined due to their interactions. Besides, it reinforces the beneficial effect of the combined effect of all tea components, as the WTEA extract showed a better preservation of sperm survival and reduced oxidative damages.

DM is responsible for several alterations in male reproductive function that impair fertility [22-24]. It was estimated that about 50% of all diabetic male individuals have some grade of subfertility and/or infertility [25]. The most prevalent form of DM is T2DM, comprising up to 95% of all diagnosed cases of DM in developed countries. The prevention of T2DM passes through the control of the prediabetic state, which usually precedes the development of T2DM (up to 10 years) and has the particularity of being reversible. Thus, if detected early, the treatment of this prodromal state, with lifestyle and drug-based interventions, may delay or even avoid the development of the disease [26, 27]. Prediabetes is characterized by resistance to an

insulin-mediated glucose disposal and a compensatory slight hyperinsulinemia [28]. Even if prediabetes does not present all T2DM features, it has several deleterious effects to human health. Prediabetes induces defects in testicular metabolism, changes in hormonal levels [24] and ionic alterations in testis and epididymis [29]. DM and prediabetes have been reported to induce irreparable damages in sperm nuclear and mitochondrial deoxyribonucleic acid (DNA), mainly due to increased OS levels in testes and spermatozoa [24, 30]. Diabetic individuals present marked reduction in sperm quality [31], including decreased sperm motility and viability [30], increased amount of spermatozoa with abnormal morphology [30, 32], and reduction in fecundity capacity [33]. After developing an animal model of prediabetes, we observed that the condition severely affected testicular and epididymal glucose metabolism, resulting in decreased lactate content and decreased sperm quality. The ingestion of a daily prepared WTEA infusion instead of water for two months prevented many of the reproductive dysfunctions induced by the disease, showing a great improvement in sperm quality [34].

In conclusion, this is the first research project reporting the beneficial effects of WTEA and its components on male reproductive function and its protective role against the deleterious effects of prediabetes in male reproduction. Although the results here presented need to be further tested in humans, the potential of WTEA as a complementary therapy to improve male reproductive health of prediabetic/diabetic men should be explored. Nowadays, many antioxidant therapies are being recommended to subfertile/infertile patients. However, there is a lack of studies regarding the effectiveness and safety of the used doses. Our data represent a step forward in the establishment of the beneficial and safe WTEA doses for male reproductive health. Besides, the use of WTEA may overcome the undesired secondary effects and habituation induced by antidiabetic drugs, which only intend to “treat” the diabetic condition regardless of the negative effects for male reproduction. The fact that WTEA is a natural product associated with a global health status and relatively inexpensive, potentiates its consumption. This project also highlights that not only the antioxidant potential, but also the combined action of all the tea components is responsible for its beneficial biological effects. The development of WTEA-based food supplements may enhance its therapeutic effect. WTEA may be a natural and effective approach to help prediabetic/diabetic men to father a child either by natural or assisted reproduction, thus decreasing the prevalence of subfertility/infertility in men with metabolic dysfunctions.

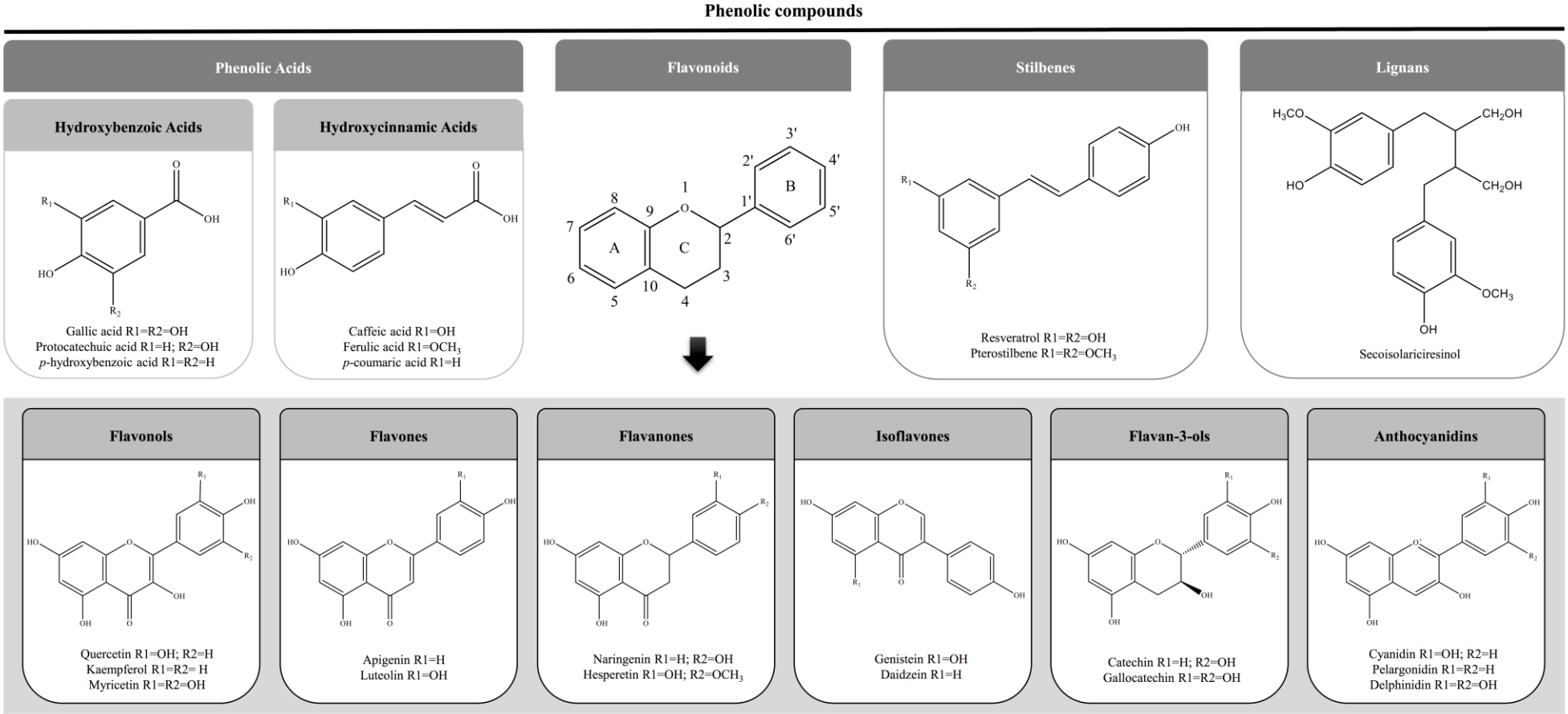
References

1. Wild S, et al. (2004) Global prevalence of diabetes estimates for the year 2000 and projections for 2030. Diabetes care 27(5):1047-53.
2. Guariguata L, et al. (2014) Global estimates of diabetes prevalence for 2013 and projections for 2035. Diabetes research and clinical practice 103(2):137-49.
3. Dias TR, et al. (2014) Sperm glucose transport and metabolism in diabetic individuals. Molecular and cellular endocrinology 396(1-2):37-45.
4. Martins AD, et al. (2013) Control of Sertoli cell metabolism by sex steroid hormones is mediated through modulation in glycolysis-related transporters and enzymes. Cell and tissue research 354(3):861-68.
5. Oliveira PF, et al. (2012) Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells. Biochimica et Biophysica Acta (BBA)-General Subjects 1820(2):84-89.
6. Levine H, et al. (2017) Temporal trends in sperm count: a systematic review and meta-regression analysis. Human Reproduction Update 23(6):646-59.
7. Barratt CL, et al. (2018) 'Man Up': the importance and strategy for placing male reproductive health centre stage in the political and research agenda. Human Reproduction 33(4):541-45.
8. Cheng TO (2004) Will green tea be even better than black tea to increase coronary flow velocity reserve? American Journal of Cardiology 94(9):1223-23.
9. Vinson JA (2000) Black and green tea and heart disease: a review. Biofactors 13(1-4):127-32.
10. Dias TR, et al. (2014) White tea as a promising antioxidant medium additive for sperm storage at room temperature: a comparative study with green tea. Journal of agricultural and food chemistry 62(3):608-17.
11. Reis M, et al. (2015) Sertoli cell as a model in male reproductive toxicology: Advantages and disadvantages. Journal of Applied Toxicology 35(8):870-83.
12. Martins AD, et al. (2014) Effect of white tea (Camellia sinensis (L.)) extract in the glycolytic profile of Sertoli cell. European journal of nutrition 53(6):1383-91.
13. Dias TR, et al. (2015) Dose-dependent effects of caffeine in human Sertoli cells metabolism and oxidative profile: Relevance for male fertility. Toxicology 328:12-20.
14. Dias TR, et al. (2017) Implications of epigallocatechin-3-gallate in cultured human Sertoli cells glycolytic and oxidative profile. Toxicology in Vitro 41:214-22.
15. Baumber J, et al. (2000) The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. Journal of Andrology 21(6):895-902.
16. Tuncer PB, et al. (2010) The effect of raffinose and methionine on frozen/thawed Angora buck (Capra hircus ancryrensis) semen quality, lipid peroxidation and antioxidant enzyme activities. Cryobiology 61(1):89-93.
17. Tremellen K (2008) Oxidative stress and male infertility--a clinical perspective. Human Reproduction Update 14(3):243-58.
18. Silber SJ (1997) The use of epididymal sperm for the treatment of male infertility. Baillière's Clinical Obstetrics and Gynaecology 11(4):739-52.
19. Sato M and Ishikawa A (2004) Room temperature storage of mouse epididymal spermatozoa: exploration of factors affecting sperm survival. Theriogenology 61(7):1455-69.
20. Sato M, et al. (2001) Prolonged survival of mouse epididymal spermatozoa stored at room temperature. genesis 31(4):147-55.
21. Dias TR, et al. (2016) The single and synergistic effects of the major tea components caffeine, epigallocatechin-3-gallate and l-theanine on rat sperm viability. Food & function 7(3):1301-05.
22. Alves MG and Oliveira PF (2013) Diabetes *Mellitus* and male reproductive function: where we stand. International Journal of Diabetology & Vascular Disease Research 1(1e):1-2.
23. Rato L, et al. (2015) Testicular Metabolic Reprogramming in Neonatal Streptozotocin-Induced Type 2 Diabetic Rats Impairs Glycolytic Flux and Promotes Glycogen Synthesis. Journal of Diabetes Research 2015, Article ID 973142.
24. Rato L, et al. (2014) Pre-diabetes alters testicular PGC1-α/SIRT3 axis modulating mitochondrial bioenergetics and oxidative stress. Biochimica et Biophysica Acta (BBA)-Bioenergetics 1837(3):335-44.
25. La Vignera S, et al. (2009) Andrological characterization of the patient with diabetes *mellitus*. Minerva endocrinologica 34(1):1.
26. Perreault L, et al. (2009) Regression from pre-diabetes to normal glucose regulation in the diabetes prevention program. Diabetes Care 32(9):1583-88.
27. Morris D, et al. (2013) Progression rates from HbA1c 6.0–6.4% and other prediabetes definitions to type 2 diabetes: a meta-analysis. Diabetologia 56(7):1489-93.
28. Association AD (2013) Diagnosis and classification of diabetes *mellitus*. Diabetes care 36(Supplement 1):S67-S74.
29. Bernardino RL, et al. (2013) Effect of prediabetes on membrane bicarbonate transporters in testis and epididymis. The Journal of membrane biology 246(12):877-83.

30. Oliveira PF, et al. (2015) White tea consumption restores sperm quality in prediabetic rats preventing testicular oxidative damage. Reproductive BioMedicine Online 31(4):544-56.
31. Amaral S, et al. (2006) Effects of hyperglycemia on sperm and testicular cells of Goto-Kakizaki and streptozotocin-treated rat models for diabetes. Theriogenology 66(9):2056-67.
32. Rato L, et al. (2013) High-energy diets may induce a pre-diabetic state altering testicular glycolytic metabolic profile and male reproductive parameters. Andrology 1(3):495-504.
33. Scarano W, et al. (2006) Sexual behaviour, sperm quantity and quality after short-term streptozotocin-induced hyperglycaemia in rats. International journal of andrology 29(4):482-88.
34. Dias TR, et al. (2016) White tea intake prevents prediabetes-induced metabolic dysfunctions in testis and epididymis preserving sperm quality. The Journal of nutritional biochemistry 37:83-93.

Annex 1

Supplementary figures



Supplementary figure 1 - Schematic illustration of the chemical structures of the main classes of phenolic compounds: phenolic acids, flavonoids, stilbenes and lignans. Phenolic acids can be further classified as hydroxybenzoic acid derivatives or hydroxycinnamic acid derivatives. The most abundant phenolic compounds in human diet are flavonoids, which can be subdivided into flavonols, flavones, flavanones, isoflavones, flavan-3-ols and anthocyanidins. Stilbenes and lignans are not widely distributed in plants.

Annex 2

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White Tea (Camellia Sinensis (L.)): Antioxidant Properties And Beneficial Health Effects

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Title: White Tea as a Promising Antioxidant Medium Additive for Sperm Storage at Room Temperature: A Comparative Study with Green Tea

Author: Tânia R. Dias, Marco G. Alves, Gonçalo D. Tomás, et al

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Title: Dose-dependent effects of caffeine in human Sertoli cells metabolism and oxidative profile: Relevance for male fertility

Author: Tânia R. Dias,Marco G. Alves,Raquel L. Bernardino,Ana D. Martins,Ana C. Moreira,Joaquina Silva,Alberto Barros,Mário Sousa,Branca M. Silva,Pedro F. Oliveira

Publication: Toxicology

Publisher: Elsevier

Date: 3 February 2015

The single and synergistic effects of the major tea components caffeine, epigallocatechin-3-gallate and L-theanine on rat sperm viability

T. R. Dias, M. G. Alves, S. Casal, B. M. Silva and P. F. Oliveira, *Food Funct.*, 2016, **7**, 1301
DOI: 10.1039/C5FO01611H

Title: White tea intake prevents prediabetes-induced metabolic dysfunctions in testis and epididymis preserving sperm quality

Author: Tânia R. Dias,Marco G. Alves,Luís Rato,Susana Casal,Branca M. Silva,Pedro F. Oliveira

Publication: The Journal of Nutritional Biochemistry

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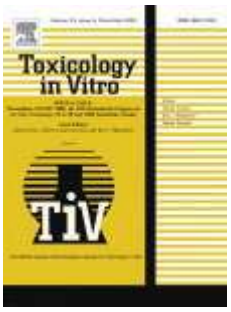

Title: Implications of epigallocatechin-3-gallate in cultured human Sertoli cells glycolytic and oxidative profile

Author: Tânia R. Dias,Marco G. Alves,Joaquina Silva,Alberto Barros,Mário Sousa,Susana Casal,Branca M. Silva,Pedro F. Oliveira

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