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Influence of bioactive compounds present in white tea in Sertoli Cells oxidative and metabolic profiles

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(Anette Sousa da Veiga)

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Resumo

O chá é a segunda bebida mais consumida em todo o mundo, sendo preparada a partir de infusão das folhas e /ou dos rebentos da espécie *Camellia Sinensis (L.)*. Existem quatro tipos de chá: verde, preto, oolong e branco. Destes, o chá branco (WTEA) permanece o menos estudado embora estudos anteriores do nosso grupo tenham demonstrado que poderá ter uma maior atividade biológica quando comparado com o popular chá verde. Com base no processamento, o WTEA pode ser classificado como: Bai Hao Yin Zhen (BHYZ), Bai Mu Dan (BMD), Gong Mei (GM) e Shou Mei (SM). Estes subtipos de WTEA diferem em qualidade, sabor e atuação sendo que BMD e BHYZ são os subtipos mais consumidos no continente asiático. O metabolismo energético é a chave para a espermatogénese, o processo de produção de espermatozoides. A glicólise anaeróbia e a fosforilação oxidativa, que ocorre nas mitocôndrias, são as principais vias metabólicas envolvidas na produção de ATP. Contudo, durante esses processos, várias fontes celulares produzem quantidades significativas de espécies reativas de oxigénio (ROS). A superprodução de ROS causa um stress oxidativo (OS), que está relacionado com vários problemas que podem resultar na subfertilidade ou infertilidade masculina. Neste trabalho, propomos estudar como os extratos aquosos de WTEA dos subtipos BMD e BHYZ podem influenciar o suporte nutricional da espermatogénese. Primeiramente, expusemos as células responsáveis por essa função, células de Sertoli humanas (hSC), a concentrações crescentes de extratos WTEA subtipos BMD e BHYZ (em mg/mL: 0.05; 0.5 e 5) para avaliar a citotoxicidade através da medição da atividade metabólica (MTT assay) e de crescimento celular (SRB assay). Apenas a exposição a 5 mg/mL de extrato de WTEA subtipo BMD induziu citotoxicidade nas hSC. Por isso, selecionamos expor as hSC a 0.5 mg/mL de extratos WTEA subtipos BMD e BHYZ durante 24h. Meios e células foram recolhidos para estudo do metabolismo recorrendo à 1H-NMR, complementado com estudos da atividade da lactato desidrogenase (LDH), mRNA e quantificação de proteínas e enzimas relacionadas ao metabolismo. Também determinamos a carbonilação proteica e o número de cópias de DNA mitocondrial nas hSC. Os nossos resultados mostram que ambos os subtipos de extrato WTEA, BMD e BHYZ aumentam os transportadores membranares relacionados à glicólise em hSC, enquanto também diminuem a atividade da LDH e expressão do complexo mitocondrial V, o que indica um mecanismo adaptativo. A carbonilação proteica também diminuiu após exposição ao extrato de WTEA de ambos os subtipos. Globalmente, estes resultados ilustram que o extrato de WTEA modula o perfil metabólico e oxidativo das hSC, o que parece ter um efeito positivo no apoio nutricional da espermatogénese. Além disso, o extrato de WTEA subtipo BMD diminuiu o OS que foi seguido pela diminuição do número de cópias de DNA mitocondrial e aumento da atividade do complexo mitocondrial II. Em geral, os nossos dados sugerem que o subtipo BMD do WTEA pode ser mais eficaz para combater os efeitos prejudiciais causados por doenças metabólicas que alteram o

suporte nutricional da espermatogênese. No entanto, mais estudos serão necessários para apoiar esta hipótese.

Palavras-chave

Células de Sertoli humanas, Chá Branco, BMD, BHYZ, Antioxidantes, Metabolismo

Resumo Alargado

O chá é uma infusão feita há mais de 5000 anos a partir das folhas e/ou rebentos da espécie *Camelia sinensis* que pertence à família Theacea. Ultrapassada somente pela água, o chá é a segunda bebida mais consumida no mundo com um consumo de aproximadamente de 120 mL/dia. O alto consumo de chá deve-se, provavelmente, ao seu elevado conteúdo em componentes fitoquímicos que promovem efeitos benéficos à saúde. Existem quatro tipos de chá: o chá preto, oolong, verde e branco. De todos, o chá branco (WTEA) é o tipo menos estudado. Com base no processamento o chá branco pode ser classificado em quatro subtipos: Bai Hao Yin Zhen (BHYZ), Bai Mu Dan (BMD), Shou Mei (SM) e Gong Mei (GM). Estes subtipos de chá diferem no seu sabor, qualidade e atuação metabólica, sendo os subtipos BHYZ e BMD os mais consumidos.

Vários efeitos benéficos para a saúde são atribuídos aos compostos fenólicos do WTEA, tais como propriedades antioxidantes, antidiabéticas e neuroprotetoras. Estas ações despoletadas pelo consumo deste chá ocorrem graças a sua interação com as vias metabólicas do organismo. Estudos anteriores demonstram que o WTEA poderá ter uma maior atividade biológica comparado ao chá verde devido ao seu maior teor em compostos fenólicos, especialmente em catequinas.

A infertilidade é uma doença do sistema reprodutor, cuja incidência tem vindo aumentar com o passar dos anos. A organização mundial da saúde define infertilidade como a incapacidade de gerar e manter uma gravidez completa após doze meses de relações sexuais regulares e desprotegidas. A causa da infertilidade poderá ter um fator masculino ou feminino, sendo que a contribuição de cada fator é compartilhada de forma igualitária. Porém existem causas de infertilidade desconhecidas, onde o fator genético e estilo de vida poderão estar relacionados.

Células de Sertoli (SC), são células responsáveis pelo suporte nutricional e físico das células germinativas em desenvolvimento, ajudando assim num correto desenvolvimento destas células, um processo denominado espermatogénese. A espermatogénese depende de uma homeostase oxidativa, uma vez que um desequilíbrio entre a produção de espécies reativas de oxigénios (ROS) e as defesas antioxidantes das células de Sertoli resultam num stress oxidativo (OS). Este stress oxidativo, causado pelos níveis excessivos de ROS contribuem para um processo de espermatogénese disfuncional que consequentemente reduz a fertilidade masculina, resultando em homens subférteis ou inférteis.

O objetivo a que nos propusemos neste trabalho foi o de estudar os efeitos de extratos aquosos de WTEA dos subtipos BMD e BHYZ em células de Sertoli humanas (hSC), focando na

influência que esta exposição das hSC aos subtipos de chá branco poderá ter a nível metabólico e oxidativo dessas células. Para tal, foram usados três grupos experimentais de biopsias testiculares: um grupo que não foi exposto ao extrato de chá (controlo), um grupo exposto ao extrato de WTEA do subtipo BHYZ e um outro exposto ao extrato de WTEA do subtipo BMD. Primeiramente foi avaliada a citotoxicidade de concentrações crescentes (0; 0.05; 0.5 e 5 mg/mL) dos extratos dos subtipos de chá branco BHYZ e BMD nas hSC através da medição da atividade metabólica (MTT assay) e crescimento celular (SRB assay). Esta avaliação permitiu selecionar a concentração de 0.5 mg/mL para exposição das hSC aos extratos de WTEA, subtipos BHYZ e BMD, durante 24h. A recolha das células e dos meios onde se encontravam, permitiu uma avaliação do perfil metabólico através da medição dos níveis de expressão mRNA dos transportadores relacionados com a glicólise, dos metabolitos recorrendo à 1H-NMR complementando com avaliações da atividade da lactato desidrogenase (LDH), quantificação de proteínas e enzimas relacionadas com o metabolismo. Avaliamos também a carbonilação proteica e o número de copias de DNA mitocondrial, bem como a atividade dos complexos mitocôndrias nas hSC.

Os nossos resultados mostram que ambos os extratos de WTEA, subtipos BHYZ e BMD aumentam a expressão dos transportadores membranares relacionados com a glicólise em hSC e diminuem a atividade da LDH, sem prejudicar a produção de lactato o que indica que o fluxo glicolítico é mantido. Além disso, a exposição das hSC aos dois subtipos de extrato reduz a carbonilação proteica evidenciando a capacidade antioxidante do chá. O complexo mitocondrial V tem a expressão diminuída em hSC expostas a ambos subtipos, apontando assim para um mecanismo de adaptação por parte destas células, face às alterações metabólicas ocorridas.

Em suma, os nossos resultados sugerem que o extrato de WTEA modula positivamente o perfil metabólico e oxidativo das hSC, contribuindo para uma espermatogénese correta e funcional. No entanto, os nossos resultados também sugerem que o extrato de WTEA, subtipo BMD será mais eficaz para combater efeitos prejudiciais induzidos por doenças metabólicas, uma vez que diminui o OS através da diminuição do ratio lactato/alanina, seguidamente com a diminuição do número de copias de DNA mitocondrial. No entanto, mais estudos serão necessários para apoiar esta hipótese.

Abstract

The tea is the second most popular drink in the world and is prepared by infusing of the leaves and/or the buds of the specie *Camellia sinensis* (L.). Tea is divided in four types: green, black, oolong and white. From those, the white tea (WTEA) remains as the less studied though previous reports from our team showed that it may have more biological activity when compared to the popular green tea. Based on the processing, WTEA can be classified as: Bai Hao Yin Zhen (BHYZ), Bai Mu Dan (BMD), Gong Mei (GM) and Shou Mei (SM). These WTEA differ in quality, flavor and performance being that BMD and BHYZ are the most consumed types grades on the Asian continent. Energy metabolism is a key for spermatogenesis, the process of spermatozoa production. Anaerobic glycolysis and oxidative phosphorylation, which takes place in the mitochondria, are the main metabolic pathways involved in ATP production. However, during those processes, several cellular sources produce significant amounts of reactive oxygen species (ROS). ROS overproduction results in oxidative stress (OS), which is related with several problems that may end up in male subfertility or infertility. Herein we proposed to study how WTEA aqueous extracts of subtypes BMD and BHYZ may influence the nutritional support of spermatogenesis. Firstly, we exposed the cells responsible for that function, human Sertoli cells (hSC), to increasing concentrations of WTEA extracts subtypes BMD and BHYZ (in mg/mL: 0.05; 0.5 and 5) to evaluate cytotoxicity by measuring metabolic activity (MTT assay) and growth (SRB assay). Only the exposure to 5 mg/mL of WTEA extract subtype BMD induced cytotoxicity to hSC. Therefore, we selected to expose hSC to 0.5 mg/mL of WTEA extracts subtypes BMD and BHYZ during 24h. Media and cells were collected for metabolism study using 1H-NMR, complemented with LDH activity and mRNA and protein quantification of metabolism-related enzymes and transporters. We also determined protein carbonylation and DNA mitochondrial copy number in hSC. Our data shows that both WTEA extract subtypes BMD and BHYZ increase glycolysis-related membrane transporters in hSC, while also decreasing LDH activity and mitochondrial complex V expression, which further indicates an adaptative mechanism. Protein carbonylation was also decreased after exposure to WTEA extract of both subtypes. Overall, these results illustrate that WTEA extract modulates metabolic and oxidative profile of hSC, which appears to have a positive effect to the nutritional support of spermatogenesis. In addition, WTEA extract subtype BMD decreased oxidative stress which was followed by decreased mitochondrial DNA copy number and increased mitochondrial complex II activity. Overall, our data suggests that WTEA BMD may be more effective to counteract deleterious effects induced by metabolic diseases that alter the nutritional support of spermatogenesis. Nevertheless, more studies will be needed to support this hypothesis.

Keywords

Human Sertoli cells, White tea, BMD, BHYZ, Antioxidants, Metabolism.

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List of Abbreviations

Acetyl-CoA – Acetyl Coenzyme A

ALT – Alanine Aminotransferase

Ap – Spermatogonia A pale

Ad – Spermatogonia A dark

BHYZ – Bai Hao Yin Zhen

BMD – Bai Mu Dan

BTEA – Black Tea

BTB – Blood-Testis Barrier

B2M – ß-2-Microglobulin

DMEM: Ham's F12 – Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12

FBS – Fetal Bovine Serum

FSH – Follicle-Stimulating Hormone

Glut 1 – Glucose Transporter 1

Glut 3 – Glucose Transporter 3

Glut 8 – Glucose Transporter 8

Glut 14 – Glucose Transporter 14

Gluts – Glucose Transporters

GTEA – Green Tea

GM – Gong Mei

hSC – Human Sertoli Cells

LDH – Lactate dehydrogenase

LH – Luteinizing Hormone

MCT1 – Monocarboxylate Transporter 1

MCT2 – Monocarboxylate Transporter 2

MCT3 – Monocarboxylate Transporter 3

MCT4 – Monocarboxylate Transporter 4

MCT – Monocarboxylate Transporters

mtDNA – Mitochondrial DNA

OTEA – Oolong Tea

OS – Oxidative stress

PBS – Phosphate buffered saline

PO – Polyphenol Oxidase

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

RONS – Reactive Oxygen and Nitrogen Species

SC – Sertoli Cells

SM – Shou Mei

SRB – Sulforhodamine B

TBS – Tris Buffered saline

WTEA – White Tea

I. Introduction

1.Tea

Camellia sinensis (L.) O. Kuntze belongs to Theaceae family. It has been used to prepare tea infusions since 5,000 years ago (Wheeler and Wheeler 2004, Dias, Tomás et al. 2013). This plant is native from southwest china, but it is cultivated in over 30 countries around the world (Lopez and Calvo 2011).

Surpassed only by water, tea is the second most consumed drink in the world (Cheng 2006) with a per capita consumption of approximately 120 mL/day (McKay and Blumberg 2002). Tea sensorial properties, low retail price, stimulating effects and potential health benefits are related to its huge consumption worldwide (Moderno, Carvalho et al. 2009, Dias, Tomás et al. 2013, Dias, Alves et al. 2014, Martins, Alves et al. 2014).

1.1 Types of Tea

Tea is an infusion prepared from leaves of C. sinensis, but each type of tea has a different composition, which depends on the types of growing conditions, geographical origin (season, climate, soil), botanical varieties and processing (de Mejia, Ramirez-Mares et al. 2009). According to the different levels of oxidation, three different types of tea can be achieved: unfermented (green and white), partially fermented (oolong) and totally fermented (black), reaction catalysed by the enzyme polyphenol oxidase (PO) (Figure 1) (Dias, Tomás et al. 2013) and commonly called “fermentation”.

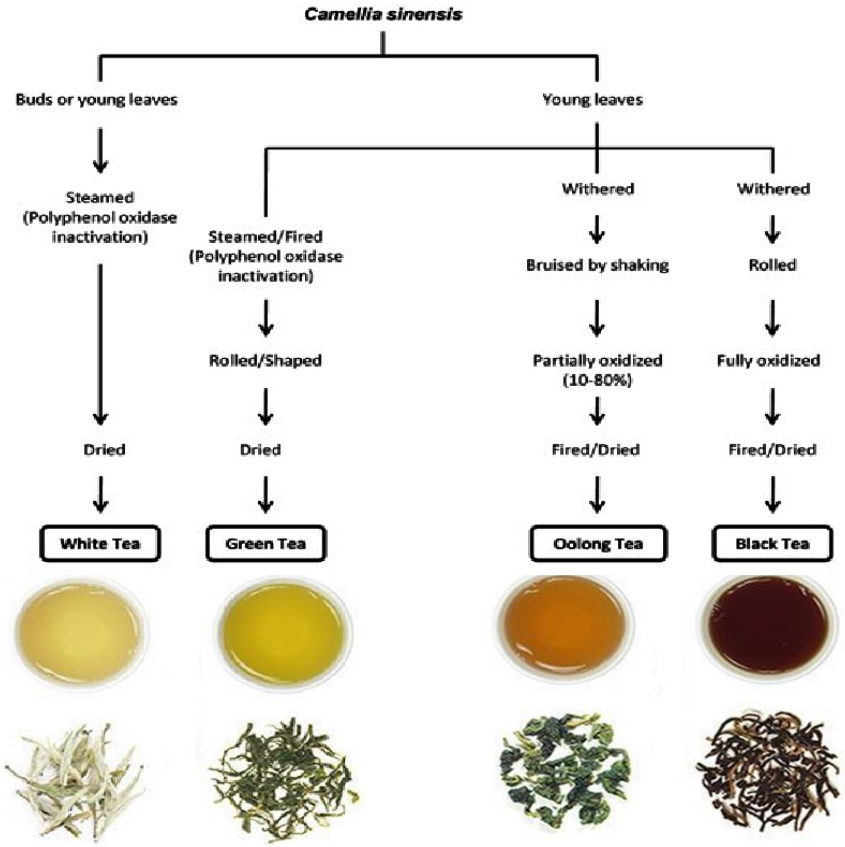


Figure 1 – Schematic representation of tea types and processing. (Adapted from (Dias, Tomás et al. 2013)).

To produce green tea (GTEA) the young leaves are steamed and rolled to minimize oxidation and to inactive polyphenol oxidase (Anderson and Polansky 2002), before drying. In black tea (BTEA), which is the mostly consumed tea type in west countries, the leaves are rolled. This rolling process disrupts cellular compartmentation and brings phenolic compounds into contact with PO and thus, the leaves suffer oxidation for 90 -120 minutes (Rusak, Komes et al. 2008). Oolong tea (OTEA) production has a shorter oxidation period comparative to black tea and has a taste and colour somewhere between green and black tea (Del Rio, Stewart et al. 2004).

The four types of teas have bioactive compounds that trigger physiologic properties such as antidiabetic (Anderson and Polansky 2002, Abolfathi, Mohajeri et al. 2012), antioxidant (Yen and Chen 1995, Costa, Magalhaes et al. 2009, Carloni, Tiano et al. 2013), anti-inflammatory (Sano, Suzuki et al. 1999, Cavet, Harrington et al. 2011), antimutagenic (Bhattacharya, Mukhopadhyay et al. 2011) and neuroprotective(Tan, Tan et al. 2003, Almajano, Vila et al. 2011). Finally, white tea the type of tea least known and thus we explore below the characteristics.

1.2 White Tea

White tea (WTEA) is originated and mainly produced at the southeast coast of China more accurately in Fujian Province, and its seasonal crop for collecting occurs only in early spring (Hilal and Engelhardt 2007). In order to attain a higher quality of tea, WTEA is prepared from young leaves or buds that are hot steam and dried immediately after picking to prevent catechins oxidation and give a bright yellow and delicate taste (Rusak, Komes et al. 2008, Dias, Carrageta et al. 2019). In this way, WTEA is considerably a nonfermented tea or lightly fermented. To reduce the formation of chlorophyll, the buds may be shielded from sunlight during growth which gives the young leaves a characteristic white appearance (Alcazar, Ballesteros et al. 2007). This complex processing of WTEA makes it rare and expensive. White tea consumption has been associated with a wide range of health benefits, such as antioxidant activity (Unachukwu, Ahmed et al. 2010) , antimicrobial(Damiani, Bacchetti et al. 2014), antielastase, anticollagenase (Thring, Hili et al. 2009), anticarcinogenic and antimutagenic properties (Song, Zhou et al. 2015, Tan, Engelhardt et al. 2017). These positive association highly contributes to the increase in tea production and exportation and the great interest in its consumption worldwide. However, WTEA remains the rarest and least processed tea and thus, its beneficial health effects and the mechanism of action are still being relatively unknown.

1.2.1 White Tea Subtypes

White tea can be obtained from three different cultivars: Big White, Narcissus White and Vegetable White. Being big white is the most popular and finest. These trees are clones obtained by selection of raw material of all white tea varieties (Sanlier, Atik et al. 2018). So, depending the cultivar and the collection standards, four grades of WTEA can be classified (Table 1).

Table 1 – White tea subtypes and characterization.

Grade	Chinese Name	English Name	Cultivar	Production	References
1st	Bai Hao Yin Zhen (BHYZ)	White tip Silver Needle or Silver needle (short name)	Big White	One bud	(Damiani, Bacchetti et al. 2014)
2nd	Bai Mu Dan (BMD) or Pai Mu Tan	White peony	Narcissus White or Big white	One bud and one or two immature leaves	(Ning, Ding et al. 2016)
3rd	Gong Mei	Tribute Eyebrow	Big White	One bud and two or three immature leaves	
4th	Shou Mei (SM)	Longetivy EyeBrow or Long-Life EyeBrow	Big white	Mature leaves	(Lin, Xia et al. 2017)

The WTEA grades are based on the tenderness of fresh tea shoots, and different grades vary in performance, flavour and taste (Pan, Jiang et al. 2018).

Bai Hao Yin Zhen (BHYZ) and Bai Mu Dan (BMD) are the most consumed white tea subtypes.

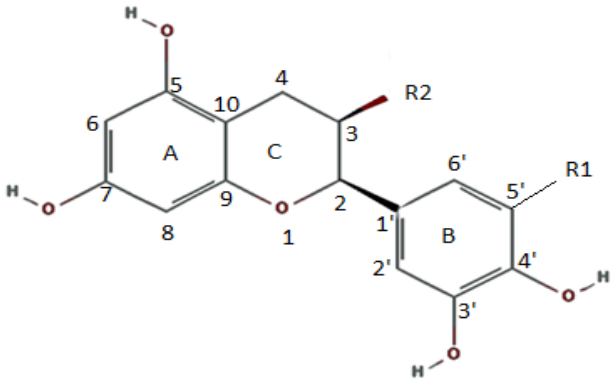
Bai Hao Yin Zhen (BHYZ) is one of the most famous, exotic and expensive WTEA subtype produced from the big white cultivar (Damiani, Bacchetti et al. 2014, Ning, Ding et al. 2016). This is made from unopened buds, which are harvested by hand (Ugochukwu, Babady et al. 2003). The first step in processing is sun drying for a day on sieves or drying mats, then baking over a slow fire until completely dried. The resultant drink is light yellow with delicate flavour (Damiani, Bacchetti et al. 2014).

White peony tea, or traditionally called Bai Mu Dan (BMD), is produced with buds and one or two immature leaves. The manufacture of BMD has a withering process under sun during 1 to 3 days, which is followed by drying in the basket. Its infusion produces a light golden-brown tea with a more intense flavour than BHYZ tea (Damiani, Bacchetti et al. 2014).

1.2.2 Phytochemical Compounds present in White tea

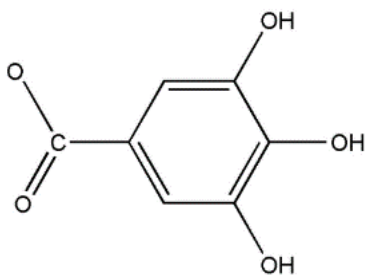
Tea has a complex chemical composition including proteins, polysaccharides, polyphenols, minerals and trace elements, amino (L-theanine) and organic acids, lignins, methylxanthines (caffeine, theophylline and theobromine), chlorophyll, carbohydrates, volatile compounds and other unidentified compounds. Some of these bioactive compounds possess health benefit.

A) Polyphenols – are the most abundant and bioactive group of compounds in tea. Flavonoids are phenolic derivatives, distributed among the plant, that have been categorized into flavonols, flavones, catechins, flavanones, isoflavonoids and anthocyanidins. Catechins or also called flavan-3-ols monomers and their gallate derivates are the major class of phenolic compounds. The major catechins present in WTEA are: (−) -epicatechin (EC), (−)- epigallocatechin (EGC), (−) -epicatechin-3-gallate (ECG) and (−) -epigallocatechin-3-gallate (EGCG). As expected, the health benefits attributed to catechins depends their chemical structure (Figure 2)



Catechin Structure

Catechins:	R1	R2
(-)-Epicatechin (EC)	H	OH
(-)-Epicatechin 3-gallate (ECG)	H	Gallate Group
(-)-Epigallocatechin (EGC)	OH	OH
(-)-Epigallocatechin 3-gallate (EGCG)	OH	Gallate Group



Gallate Group

Figure 2 - Chemical structures of the main tea catechins. Illustration of two aromatic rings (A, B) and a dihydropyran heterocyclic ring (C), which is the basic structure of the flavonoids. The (-)- epicatechin (EC) is constituted by an ortho-di-hydroxil group in the B ring (at carbons 3' and 4') and a hydroxyl group in the C ring (at carbon 3), and its ester derivative (-)-epicatechin 3-gallate (ECG) differs of this structure by possessing an additional gallate moiety esterified in the C ring, at carbon 3.

Catechins have two aromatic rings (A-ring and B-ring) connected to a dihydropyran heterocycle (the C-ring) (Braicu, Ladomery et al. 2013). The chemical differences in catechins are due to the different group attached in those rings. The EC has an ortho-dihydroxyl group in the B-ring (at carbons 3' and 4') and a hydroxyl group on the C-ring (carbon 3'), EGC has a trihydroxyl group (at carbon 3', 4', and 5') on B-ring , ECG contains a gallate moiety esterified on the C-ring (at carbon 3'). EGCG differs by possessing trihydroxyl group on the B-ring (at carbon 3', 4', 5') and a gallate moiety esterified (at carbon 3') on the C-ring. (Graham 1992, Braicu, Ladomery et al. 2013, Dias, Tomás et al. 2013). White and green tea are the types which have the highest content of catechins.

The black and oolong tea has minor quantities of catechins, once during its processing they are oxidized by PO and originate complexes such as thearubiginis, theaflavins, theaflavinic acid and proanthocyanidin polymers.

Theaflavin, theaflavin 3 – monogallate, theaflavin 3'- monogallate or theaflavin 3'- gallate and theaflavin 3,3'- digallate are characterized by the benzotropolone ring structure (Mukhtar and Ahmad 1999, Tan, Engelhardt et al. 2017). That bright red-orange pigment contributes not only to the colour of tea but also to the bitterness/taste and astringency of oolong and black tea (Dias, Carrageta et al. 2019). The oxidized theaflavins originated thearubigins that are heterogenous in nature, have higher molecular weight and significantly contributed towards taste and colour (Obanda, Owuor et al. 2004, Karori, Wachira et al. 2007). The thearubigins are poorly characterized chemically (da Silva Pinto 2013). The theaflavins and thearubigins are formed by oxidation process and therefore it is believed that both are detected in significative quantities in black and oolong tea. Usually there is an assumption that these pigments increase while catechins decrease (Tan, Engelhardt et al. 2017) though it is not clear why this occurs.

In general, the beneficial effects of tea are ascribed to the amount of catechins which have antioxidant, antimicrobial, antidiabetic and other health promoting properties. The EGCG is the catechin better characterized and is the most abundant catechin present in WTEA representing 50-80% of the total and thus, responsible for most of the positive catechins effects described (Khan and Mukhtar 2007).

Up till now, it is not clear between BMD or BHYZ which has the highest amount of catechins, since the polyphenolic composition of different teas is a result of the leave processing and the level of oxidation during manufacturing. However some authors report that BHYZ is the sub-type of white tea with the largest amount of polyphenols (for review read (Ning, Ding et al. 2016, Yang, Hu et al. 2018)) since it is in the first grade of WTEA sub-types. But others authors argue that BMD is the one that contains more catechins (Pan, Jiang et al. 2018). Thus, this is a controversial field of research that has been somewhat overlooked in the last few years.

B) Amino acids – theanine or chemically 5-N-ethylglutamine, is the most abundant amino acid in tea. It is formed from glutamic acid and ethylamine, synthesized in the root of tea by theanine synthetase and accumulated in the leaves (Keenan, Finnie et al. 2011, Adhikary and Mandal 2017). Theanine is a non-proteinogenic amino acid present (Keenan, Finnie et al. 2011) in tea with a 50% presence compared with the other free amino acids (Horanni and Engelhardt 2013, Adhikary and Mandal 2017). Whith the increase of leaves maturity there is a reduction of theanine (Ning, Ding et al. 2016, Liu, Wu et al. 2017), whereas it suffers hydrolysation through exposure to sunlight and heat producing their precursor ethylamine, which is used in the synthesis of catechins (Vuong, Bowyer et al. 2011). The amount of theanine in WTEA is similar in green tea, the oolong tea is the type of tea with minor quantity of this compound (Keenan, Finnie et al. 2011, Horanni and Engelhardt 2013). Theanine is responsible for the unami taste of the tea. This compound has been considered a relaxant agent often used to reduce the blood pressure (Juneja 1999, Rogers, Smith et al. 2008, Cooper 2012). However, the health benefits of theanine are not limited to the relaxing effects but also presents several other health promoting benefits including antidiabetic (Zheng, Sayama et al. 2004, Kinuyo, Shuhei et al. 2005) and antioxidant effects as well as preventing memory degeneration (Egashira, Ishigami et al. 2008, Haskell, Kennedy et al. 2008) and immune system improvement (Kurihara, Shibahara et al. 2007, Miyagawa, Hayashi et al. 2008). According to studies by others, BHYZ white tea sub-type has a higher theanine content when compared to the sub-type BMD(for review read (Tan, Engelhardt et al. 2017, Yang, Hu et al. 2018). However, other groups showed very distinct, even opposite, results indicating that white tea subtype BMD has higher theanine content than BHYZ subtype (for review read (Pan, Jiang et al. 2018)). Thus, there is some controversy on the chemical composition of each white tea subtypes, further highlighting the need for research on this field.

C) Methylxanthines (Theobromine, caffeine and theophylline) – are a group of purine-like alkaloids present in food sources such as coffee, cocoa and tea (Ashihara, Kato et al. 2011). Caffeine (1,3,7-trimethylxanthine) represents the major methylxanthine present in the tea followed by theobromine (3,7-dimethylxanthine), having a percentage of 2-3% dry weight (Ashihara, Kato et al. 2011). In immatures leaves and buds there is an

accumulation of caffeine and theobromine because the synthesis of these methylxanthines occurs in a constitutive way in those regions, which may indicate that BHYZ will be the subtype of WTEA with higher amount of methylxanthines (Ning, Ding et al. 2016). Theophylline origin from catabolism of caffeine, however it is hardly mensurable in tea because it is rapidly degraded by CO_2 (Ashihara and Kubota 2006, Ashihara, Sano et al. 2008). It has been shown that WTEA has higher amount of methylxanthines than GTEA (Unachukwu, Ahmed et al. 2010) thus promoting a higher interest for WTEA since methylxanthines contribute to several beneficial health effects in human diseases. For instance, a moderate consumption of caffeine is linked to weight loss since it increases metabolism ratio; it also reduces the risk of developing neurodegenerative disease and some cancers (Cano-Marquina, Tarin et al. 2013, O'Keefe, Bhatti et al. 2013, Gonzalez de Mejia and Ramirez-Mares 2014). Caffeine acts at the level of the cellular metabolism, according to a recent study of our group (Dias, Alves et al. 2015), showing that moderate caffeine consumption appears to have a positive effect for male reproductive health. In that work, SCs exposed to a moderate concentration of caffeine (50 μM) presented an increase in lactate production and glucose transporter expression which promoted to a positive effect in the nutritional support of germ cells and their development (for review read (Dias, Alves et al. 2015)). Caffeine also acts on the central nervous system, particularly by competitively inhibiting the action of adenosine in cells by blocking their receptors. That action results in increases released of some hormones like serotonin, norepinephrine and dopamine (Nawrot, Jordan et al. 2003). It has been shown that excessive consumption of caffeine leads to adverse effects, and in an adult the symptoms may range from insomnia, irritability, cardiac arrhythmia to a gastrointestinal disorders (Nawrot, Jordan et al. 2003). Thus, it is crucial to have caffeine intake controlled and followed. Further studies are needed to unveil the positive and negative effects of caffeine due to tea intake and the mechanism of action responsible for such effects.

1.3 Potential effects of White Tea

Since thousands of years, plants have been used for therapeutic purposes as it is known that their phytochemicals have several health promoting properties. Indeed, with the advancement of technology compelling evidence has shown the positive effects of tea consumption on health by ameliorating some clinical features of diseases and preventing the rapid progression of diseases (Lorenzo and Munekata 2016).

Tea is consumed since very ancient times, and its consumption related to beneficial effects that are not fully clarified, particularly its mechanisms of action. Thus, it is crucial to study the effects of tea consumption on human body, in order to ensure a safe consumption,

elucidate its mechanism of action and elucidate how it can become part a complete healthy diet.

1.3.1 Antioxidant Potential

The antioxidant potential of natural compounds and mixtures has been gaining momentum and increasing interest due to their capacity to scavenge free radicals, reducing the adverse effects of reactive oxygen and nitrogen species (RONS)(Kurutas 2016).

Most living organism have an efficient enzymatic or non-enzymatic system defence against excessive reactive oxygen species (ROS) production. However, several factors, such as lifestyle (i.e. diet, smoking, alcohol consumption, drugs addiction among others) and internal factors (Dias, Carrageta et al.) can decrease the efficiency of endogenous antioxidant defences, generating a decline in the redox balance towards an inability to supress ROS (Rietveld and Wiseman 2003). Chronic exposure to ROS and reactive nitrogen species (RNS) cause damage to DNA, lipidic membrane, lipoproteins and at functional and structural level of proteins. It affects several organs systems promoting deleterious health effects. For instance, oxidative stress is present in more than half of infertile or sub-fertile men (Tremellen 2008), and thus, the use of antioxidants that can scavenge the RONS is useful to prevent the progression or onset subfertility/infertility due to oxidative stress.

Oxidative stress is known to cause germ cell damage by conditioning male fertility, since it impairs antioxidant defences in the male reproductive system. Several studies have shown that tea has high antioxidant properties due to its high content in catechins that act as free radical scavengers (Nakagawa and Yokozawa 2002, Yilmaz 2006) and metal chelators (Soobrattee, Neergheen et al. 2005). Studies have also shown that tea catechins and polyphenols are physiologically effective in the *in vitro* abduction of ROS and RNS, including radicals peroxyl, singlet oxygen (Guo, Zhao et al. 1999), superoxide (Nanjo, Honda et al. 1993, Nakagawa and Yokozawa 2002) and hypochlorous acid (Scott, Butler et al. 1993). This antioxidant process usually acts on the elimination of radicals that are in the chain of lipid peroxidation reactions (Tsao 2010). That is, they interrupt chain reactions and give electrons to radicals making them less reactive and more stable (Rice-Evans, Miller et al. 1996, Guo, Hsieh et al. 2009). Another form of action is through metal chelation in which generally that action falls on transition metals such as iron (Fe^{2+}) which thus prevent oxidation from hydroxyl radicals that are highly reactive (Pietta 2000, Perron and Brumaghim 2009).

The number and the position of hydroxyl groups in molecules influence the antioxidant capacity of polyphenols (for review read (Braicu, Ladomery et al. 2013)). The structure appears to be important for those antioxidant function including: ortho 3'-,4'-dihydroxyl (catechol) group in B-ring, that promotes a stable phenoxyl radical due to effective electron delocalization (Wiseman, Balentine et al. 1997) or the 3',4',5' – trihydroxyl (gallate) group in the B-ring, a

gallate group esterified at the 3 position of C-ring, and hydroxyl groups at the 5 and 7 positions of the A-ring (Rice-Evans, Miller et al. 1996).

A study in male rats conducted by Kalender and his colleagues demonstrated the protective antioxidant effect of polyphenols, especially the catechins, in testis after exposure to a pesticide. After analysis of antioxidant enzymes and lipidic peroxidation, the authors report that polyphenols successfully improved the activity of the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione -S-transferase in addition to reducing the content of malondialdehyde and ameliorate the histopathological examination. In sum, this study showed that rats subjected to exposure to pesticide plus catechins present an increase in antioxidant defences and also their activity when compared with the rats just exposed to pesticide. In addition, the joint treatment with catechins was able to avoid degenerative damage of the testis as evaluated by histology (for review read (Kalender, Kaya et al. 2012)).

To determine the bioactive potential and the component of tea with interest for disease prevention, it is essential to study the pharmacokinetic, distribution, absorption, metabolism and excretion of tea and its phytocomponents (Wheeler and Wheeler 2004). Polymers, esters and glycosides are tea abundant component but are not always absorbed by oral administration. The effectivity of the compound are not only correlate on the amount ingested, but on the bioavailability (Holst and Williamson 2008). Some clinical trial has demonstrated after 30 to 60 minutes of single dose ingestion of tea improves plasma antioxidant capacity of healthy adults (Benzie, Szeto et al. 1999, Leenen, Roodenburg et al. 2000, Sung, Nah et al. 2000). Koutelidakis and collaborators (2009) reported that supplementation of WTEA extract for five consecutive days increase the antioxidant capacity plasma but also in different organs in mice such lungs and heart (for review read (Koutelidakis, Argyri et al. 2009)). Theaflavins, flavonol glycosides and the catechins, specially EGCG, are also thought to be the responsible for antioxidant properties of tea. The antioxidant capacity depends of tea variety and EGCG content (Hilal and Engelhardt 2007). Several studies in animals, epidemiological studies and *in vitro* studies conducted reached the conclusion that WTEA has potentially protective effects for many health problems.

1.3.2 Neuroprotective Potential

The brain is very sensitive to oxidative stress because it has a low antioxidant system and a high oxygen content in this region that easily reacts with oxidizable fatty acids (Wang and Michaelis 2010). Therefore, oxidative stress has been related to neuronal death, consequently leading to neurodegenerative disorders including Parkinson (Surendran and Rajasankar 2010), Huntington (Stack, Matson et al. 2008) and Alzheimer disease (Agostinho, Cunha et al. 2010, Dumont, Lin et al. 2010). A study conducted by Almajano, Vila and Gines demonstrated the neuroprotective effects of WTEA on oxidative stress induced in striatal cells.

Those cells were induced to stress oxidative and exposure to WTEA significantly increased cell survival (for review read (Almajano, Vila et al. 2011)). The neuroprotective of WTEA is usually associated with its high polyphenolic content, mainly of catechins and other flavonols (Almajano, Carbó et al. 2008). The easy ability of EGCG to cross the brain barrier in mice and reach the brain parenchyma has been reported (Suganuma, Okabe et al. 1998). In addition, the neuroprotective effect of the WTEA extract on hydrogen peroxide induce toxicity in PC12 cells (Lopez and Calvo 2011). These cells were treated with different doses of WTEA (10-250 µg/ml) and cell survival after treatment with WTEA was significantly higher when compared with cells non-treated with WTEA. Thus, WTEA extract has demonstrated an excellent antioxidant effect with capacity to reduce oxidative stress in those cells. On the other hand, the neuroprotective effects of tea as not only been associated to the polyphenols contribution but also caffeine has demonstrated neuroprotective effects (Cunha 2005, Duarte, Carvalho et al. 2009). L-theanine is ascribed to induce relaxation feeling by lowering cortisol levels and reducing psychological and physiological stress (Kimura, Ozeki et al. 2007). In sum, these reports discussed suggest that the protection induced by tea consumption may come from the synergetic interaction among the various components of tea and not for a single phytocomponent.

1.3.3 Antidiabetic Potential

Diabetes is a disease whose prevalence has been increasing in the last decades. The search for new therapies or drugs from natural products, as a complementary therapy, has been increasing with the aim to reduce the side effects of current drugs and the harmful effects of disease. Some evidence indicates tea as a hypoglycaemic agent (Mackenzie, Leary et al. 2007),though the mechanism responsible for this effect is not yet fully clarified. According to a study by Islam performed in diabetic induced rats, the treatment with WTEA extract has a positive on insulin sensitivity by increasing body sensitivity. The hepatic synthesis of glycogenic can also improve after the WTEA extract consumption by diabetic rats illustrating a mechanism by which WTEA consumption promotes antidiabetic activity (for review read(Islam 2011)). Hyperglycaemia is also associated with high increase of glucose oxidation promoting oxidative stress which in turn has been strongly associated with several diseases, including cardiovascular diseases (Teresa Vanessa, Annamaria et al. 2013). Alves and colleague (Alves, Martins et al. 2015) demonstrated in study conducted on prediabetic rats that the consumption of WTEA improves the glycolytic and oxidative profile of the cardiac tissue. Rats that consumed WTEA for two months presented lower glucose intolerance and higher insulin sensitivity, as well as an improved cardiac oxidative state (Alves, Martins et al. 2015). Thus, the antidiabetic potential for WTEA consumption has been consecutively demonstrated, with relevant improvements in several organs.

White tea has exhibited antidiabetic effects by reducing the oxidative stress as discussed, but improvements were also described regarding hyperlipidemia reported in diabetic

individuals. Söhle and collaborators reported the effects of WTEA extract on human subcutaneous preadipocytes cultures, where they observed an lipolysis induction and an adipogenesis inhibition clarifying the anti-obesity effects of this natural products (Söhle, Knott et al. 2009)

Diabetes is associated with increased RONS formation and decreased antioxidant potential (Rahimi, Nikfar et al. 2005). Increased oxidative stress has been suggested to be the major cause of hyperglycemia which triggers many of the knowm diabetic complications (Valko, Leibfritz et al. 2007). Studies have shown that EGCG ameliorate cytokine-induced ß-cell damage *in vitro* (Han 2003) and prevents the decrease of cellular mass induced by treatment with multiple low dose of streptozotocin *in vivo* (Song, Hur et al. 2003).

A recent study by our group (Dias, Alves et al. 2016) performed on prediabetic rats showed that WTEA intake ameliorates the metabolic alterations cause by the disease in testis and epididymis. The WTEA intake by prediabetic induced rats allows an adjustment of testicular metabolism to maintain the normal levels of energetic supplies and also improves sperm motility and viability up to normal levels. The discussed reports illustrate that WTEA can be a good candidate to ameliorate the diabetes-induced deleterious effects.

2. Sertoli cells

2.1 General Aspects

Sertoli cells were originally described by Enrico Sertoli, at 1865, and assure several important functions to male fertility. They are arranged in a columnar shape, with long and slim mitochondria and their cytoplasm presents a lipid droplet (Bawa 1963, Schulze 1974). The nuclei of these cells have an irregularly shape, but normally they are oval. The dimensions of SC permits the support more than one germ cell (Alves, Rato et al. 2013).

The SC are responsible for spermatogenesis regulation and the amount of spermatozoa produced (Walker and Cheng 2005). These cells are often called “nurse cells” (Foley 2001) and their functions are not limited to the development of functional testis but also express male phenotype (Sharpe, McKinnell et al. 2003, Mruk and Cheng 2004). They perform several functions in the male reproductive system such as: a) nutritional and physical support to the development germ cells; b) phagocytosis of residues and degenerative or apoptotic germ cells; c) production and control of components of seminiferous tubular fluids (Feig, Bellvé et al. 1980, Jutte, Jansen et al. 1982, Griswold 1995, Johnson, Thompson et al. 2008). Indeed, the SC represent the main somatic cells of the tubular compartment of the testes.

The testes are the primary complex reproductive organs, suspended in the scrotum by the spermatic cords (Moreira, Monteiro et al. 2017) and with two essential functions: production of spermatozoa and sex hormones (Mikos, Sarakinos et al. 1993, Foley 2001, Rato, Socorro et al. 2010). The testicular parenchyma is composed by interstitial tissue and seminiferous tubule (Figure 3-A), and is surrounded by two tunics, the outer tunica is the tunica vaginalis and the tough fibrous outer membrane is called the tunica albuginea (Figure 3 -B). Each testis is divided by a septum connective tissue into about 250-300 lobules each on containing 1-3 convoluted seminiferous tubules (Moreira, Monteiro et al. 2017).

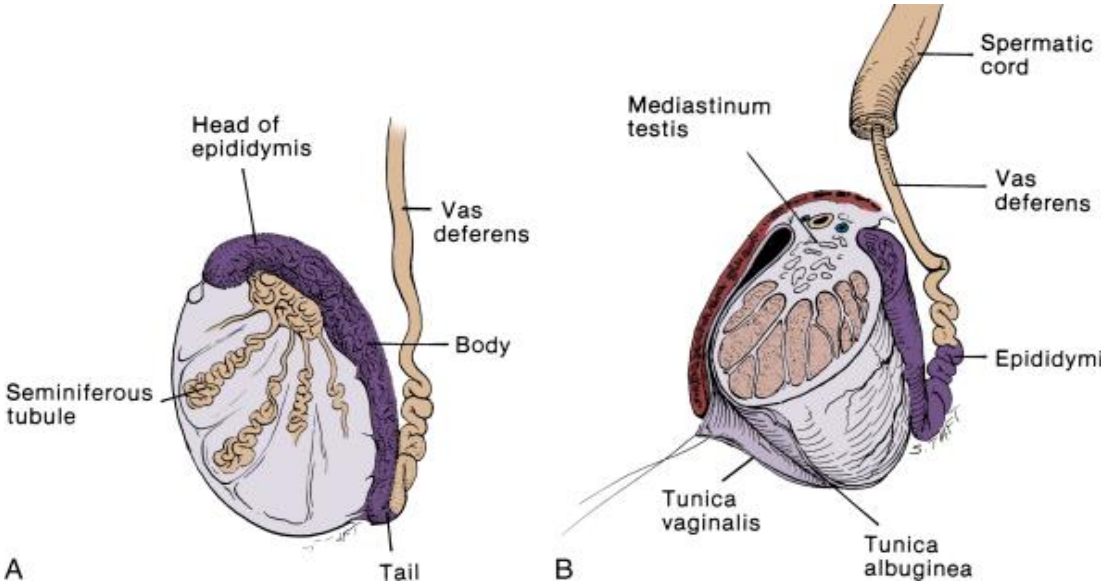


Figure 3 – **Schematic illustration of the testis and epididymis. A -**One to three seminiferous tubules fill each compartment and drain into the rete testis**.** Efferent ductules become convoluted in the head of the epididymis and drain into a single coiled duct of the epididymis. **B** - Cross section of the testis, showing the mediastinum and septations continuous with the tunica albuginea. The parietal and visceral tunica vaginalis are confluent where the vessels and nerves enter the posterior aspect of the testis (Brooks 2007).

Generally, there are about 600 seminiferous tubules in the human testis, organized as longitudinally oriented coiled that arranged in funnel shape geometry and stacked within each other (figure 3- A). The rete testis is responsible for transporting the spermatozoa from seminiferous tubules into efferent ducts being that this structure is formed by joined short areas of transitional epithelium at the end of each seminiferous tubule.

Interstitial tissue normally contains the blood and lymphatic vessels, which are important for the movement of hormones and nutrients into and out the testis (O'Donnell, Robertson et al. 2001). The Leydig cells are located within the interstitial compartment and since 1903 their endocrine role in the control of male sexual characteristics was disclosed (Bouin P. 1903). These cells secrete testosterone (T), which is the main important hormone to the development of male sexual characteristic and an effective male reproductive function (Dohle, Smit et al. 2003, Martin and Tremblay 2010).

The seminiferous tubules are a tubular compartment, which it is responsible for 65-80% of the testicular volume and contains germ cells, SC and the peritubular myoid cells (PMCs). Adjacent SC form tight junctions with each other establishing the blood-testis barrier (BTB) and thus providing an immunoprivileged microenvironment suitable to the development of germ cells, the tight junctions are dynamic and responsible for controlling the movement of nutrients and solutes , in such a way that nothing larger than 1.000 Da can pass from the outside to the inside (Walker and Cheng 2005) and preventing the entrance of molecules that could reach the germ cells. BTB divides the seminiferous epithelium into the basal compartment where spermatogonia and spermatocytes are found; and apical (adluminal) compartment where are found meiotic spermatocytes, round spermatids, elongated spermatids and spermatozoa (Mruk and Cheng 2004, Su, Mruk et al. 2011).

2.2 Spermatogenesis in brief

Spermatogenesis is the complex maturation process of germ cells, that undergo proliferation, differentiation and meiosis to generate haploid elongate spermatozoa. For the successful process to occur within the seminiferous tubule, it is necessary a near association of germ cells with Sertoli cells. The spermatogenesis takes place within seminiferous epithelium, starts at puberty and continues throughout the entire human life. The development of the germ cells is a process complex, extremely well regulated and divided in three steps, mitosis, meiosis and spermiogenesis (O'Donnell, Robertson et al. 2001, Lie, Mruk et al. 2010).

Spermatogonia are undifferentiated cells that undergo either self-renew or differentiation. They are in the basal compartment of the seminiferous tubule and in direct contact with SC (Neto, Bach et al. 2016). There are three models of spermatogonia self-renewal: spermatogonia type A which has two subclasses A dark (Ad) and A pale (Ap), and spermatogonia B (Clermont 1966). The Ap spermatogonia type are committed to divide to give rise to type B spermatogonia, as well as, to renew their own population, Ad spermatogonia are the non-proliferative reserve of spermatogonia population, under testicular damage they may be capable to transition to Ap (Clermont 1969, van Alphen and de Rooij 1986, van Alphen, van de Kant et al. 1988). The type B spermatogonia are those that differentiate in primary spermatocytes. Preleptotene spermatocytes enter the meiosis and after the first meiosis the cell contains only a pair of homologous chromosomes becoming secondary spermatocytes that are in the adluminal part of the seminiferous tubule. The last stage of spermatogenesis is spermiogenesis or also called haploid (Russel et al.1993), which includes various differentiation events culminating in differentiated spermatozoon. Some of the changes that occur in spermatids are the formation of acrosome, flagella, nucleus condensation and its elongation (Russell and Peterson 1984, de Kretser, Loveland et al. 1998).

This complex process is regulated by multiple hormones (O'Donnell, Robertson et al. 2001). The regulation begins at hypothalamus which secretes the gonadotrophin-release

hormone (GnRH) which in turn stimulates the release of two gonadotropins the Luteinizing hormone (LH) and Folicle-stimulating hormone (FSH) that act in the testis levels. The hypothalamus-pituitary-gonadal axis (HPG) is essential for the regulation of secondary sexual male characteristic, spermatogenesis and fertility, so any dysfunction on this axis may lead to infertility or a reduced fertility rate.

At testicular level, the glycoprotein hormone, LH, stimulates Leydig cells to produce testosterone (T) (McLachlan 2002), which is crucial to a normal spermatogenesis. Studies in LH receptor knockout mice showed that the animals have incomplete spermatogenesis, low testosterone levels and absent mature Leydig cells. However, testosterone treatment was able to increase testis size and improve fertility, though the animals remained infertile as only 9% of fertilization attempts resulted in pregnancy (Pakarainen, Zhang et al. 2005). In humans, mutations in LHß result in low amounts of testosterone, absence or delayed puberty and men with oligo- or azoospermia (Axelrod, Neer et al. 1979, Weiss, Axelrod et al. 1992, Lofrano-Porto, Barra et al. 2007). A recent study demonstrated that men with hypogonadism caused by mutations in LH, even after exogenous administration of LH do not improve male sexual characteristics and continue to be infertile (Basciani, Watanabe et al. 2012).

FSH also belongs to the family of glycoproteins hormones and is involved in the regulation of development, growth and reproductive functions (Grigorova, Punab et al. 2010). Receptors of this hormone in testis are found exclusively in SC (Alves, Rato et al. 2013), regulating the proliferation of these cells and the development of the testis (MCLACHLAN, O'DONNELL et al. 2002, Walker and Cheng 2005). FSH acts on initial events of spermatogenesis, stimulating the proliferation of spermatogonia. Studies showed that FSH deprivation in monkeys results in a reduction of spermatocytes, as spermatogonia have more difficulty in differentiating themselves. In addition, sperm output in the ejaculate also decrease, which led the authors to conclude that spermiogenesis was severely affected in those animals (Aravindan, Gopalakrishnan et al. 1993). Therefore, any dysregulation of these hormones leads to a dysfunctional spermatogenesis and can cause various levels of problems in male fertility.

2.3 Sertoli cells Metabolism

Sertoli cells are important for the progress of germ cells, providing physical and nutritional support (Lee, Richburg et al. 1999). These cells have the ability to metabolize many substrates but use preferentially glucose, which is metabolized to pyruvate that can then be converted to other metabolites, such as lactate, which is the preferred germ cell substrate (Riera, Galardo et al. 2009, Dias, Martins et al. 2013, Mateus, Feijo et al. 2018).

Glucose transporters (Gluts) are responsible for the passive transport of glucose without energy consumption, thus allowing it to enter to the cell (Mueckler 1994, Riera, Galardo et al. 2009) . Gluts are a family of structurally related glycoproteins. To date, 14 glucose transporters

isoforms have been identified, Glut 1 to Glut 14 (Manolescu, Witkowska et al. 2007). In SC four Gluts (Glut 1, Glut 2, Glut 3 and Glut 8) have been identified (Angulo, Rauch et al. 1998, Carosa, Radico et al. 2005, Galardo, Riera et al. 2008, Oliveira, Alves et al. 2011, Oliveira, Alves et al. 2012, Martins, Moreira et al. 2015). Glut 1, Glut 2 and Glut 3 are present in plasma membrane and thus are proposed to play a crucial role in the incorporation of glucose from the extracellular medium to the SCs (Angulo, Rauch et al. 1998, Galardo, Riera et al. 2008, Riera, Galardo et al. 2009, Oliveira, Alves et al. 2012, Martins, Moreira et al. 2015). Glut 8 should not play a very relevant role in glucose transport, as it is not located in the plasma membrane (Piroli, Grillo et al. 2002).

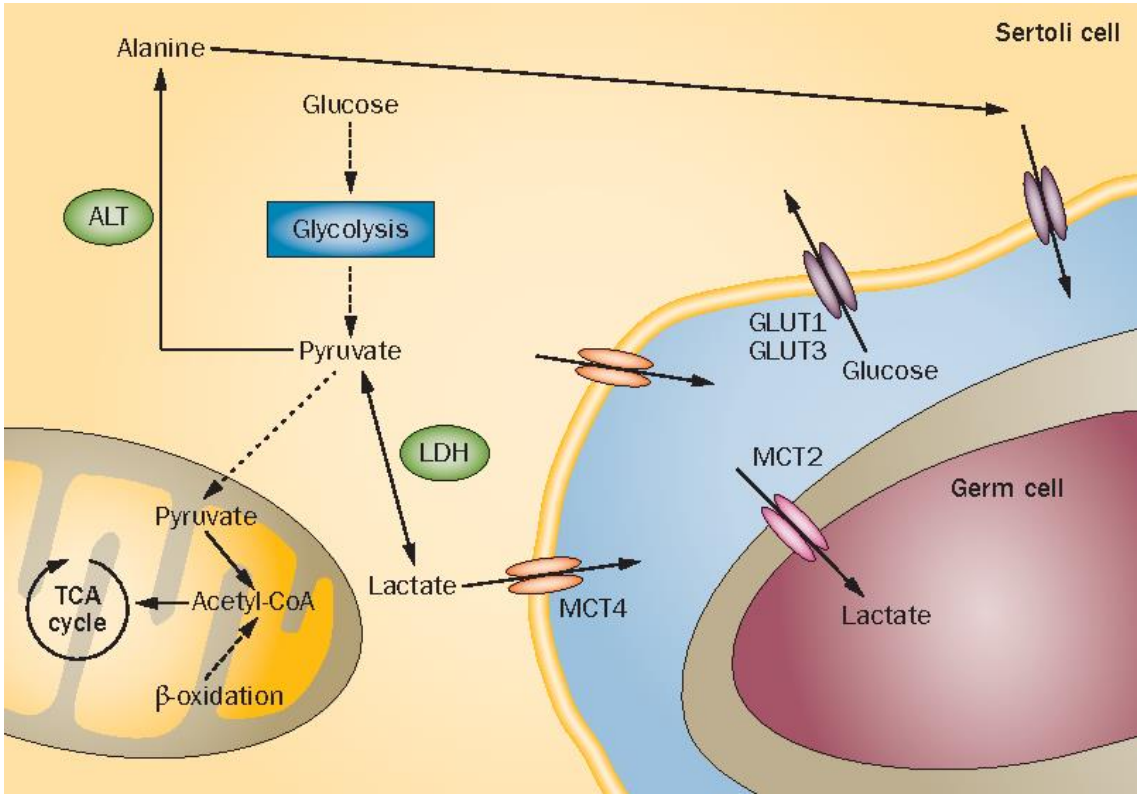


Figure 4 - Schematic illustration of Sertoli cells metabolism. Sertoli cells (SCs) are capable of consuming a variety of fuels including glucose, lactate and fatty acids. Abbreviations: ALT- Alanine aminotransferase; Glut 1 – glucose transporter 1; Glut 3 – glucose transporter 3; LDH – lactate dehydrogenase; TCA – tricarboxylic acid; MCT2 – monocarboxylate transporter 2 and MCT4 – monocarboxylate transporter 4. Image from (Rato, Alves et al. 2012).

Within the cell, glucose undergoes the glycolytic process turning into pyruvate after a few reactions. The pyruvate originated at the end of the glycolysis can be further processed in three predominant pathways: a) transported to mitochondria matrix to form acetyl coenzyme A (acetyl-CoA) b) be converted to lactate by the enzyme lactate dehydrogenase (LDH) or c) be converted to alanine by the alanine aminotransferase enzyme (ALT) (Figure 4) (Dias, Martins et al. 2013, Gray, Tompkins et al. 2014, Oliveira, Martins et al. 2015). However, the metabolic pathway is adjusted according to the needs of the cells. The conversion of pyruvate to lactate

is a preferred way during spermatogenesis, as lactate is the substrate of selection for germ cells.

The LDH isoenzymes are encoded by a multigenic family. In mammals they are encoded by three loci: LDHa, LDHb, LDHc, being that the last is only expressed in the testis (Boussouar and Benahmed 2004), with a significant expression in spermatids and sperm (Li, O'Brien et al. 1989). LDH becomes a regulator of germ cell development and metabolism, since its action in the production of lactate is crucial for their survival due to the anti-apoptotic action and the energetic dependence of this substrate.

The acetate and lactate produced can be released in to the intratubular fluid through proton/ monocarboxylate transporters (MCT), mainly monocarboxylate transporter 4 (MCT4) (Figure 4) (Boussouar and Benahmed 2004, Oliveira, Martins et al. 2015). These transporters catalyse facilitated lactate diffusion along with a proton (Brauchi, Rauch et al. 2005, Halestrap 2012). Monocarboxylate transporters (MCT) belong to the family of transporters coded by the SLC16 family gene, composed of 14 members, however only MCT 1 to MCT4 are characterized functioning as proton-linked MCT (Halestrap 2012). The MCT1 , MCT2 and MCT4 isoforms are extensively expressed by various tissues while MCT3 isoform is specifically expressed in the retina (Philp, Yoon et al. 2001, Brauchi, Rauch et al. 2005, Jones and Morris 2016).

II. Objective of the project

Infertility is a disease of reproductive tract that has been increasing over the years. The World Health Organization defines infertility as the incapacity to generate and maintain a full-term pregnancy after 12 months of regular and unprotected sexual intercourse. The cause of infertility can be due to male or female factor, and contribution from each side is suggested to be approximately shared in equals parts.

Sertoli cells are main responsible for a successfully development of the germ cells, a process called spermatogenesis. Oxidative stress (OS) caused by an unbalance between the antioxidant defences and the reactive oxygen species (ROS). Excessive levels of ROS production contributed to a dysfunctional spermatogenesis and consequently to subfertility/infertility in men.

Tea is one of the most worldwide consumed beverages, and its consumption has been associated with beneficial effects to human health, such as neuroprotection, antidiabetic and antioxidant. However, there are several types and subtypes of tea. Only few studies have been focused on the effects of WTEA, and even less on the effects and mechanisms of action Bai Hao Yin Zhen (BHYZ) and Bai Mu Dan (BMD) subtypes, the most consumed.

The main purpose of this work is to study the effects of BHYZ and BMD WTEA subtypes, in the metabolism and bioenergetics of human Sertoli cells (hSC) by analysing the oxidative and metabolic profile of these cells. In addition, we also aim to compare the possible distinct beneficial effects promoted by BHYZ and BMD to the nutritional support of the spermatogenesis by hSC. Under, this perspective first we selected a concentration of white tea extract from both subtypes without cytotoxicity to human Sertoli cells by viability testing (SRB and MTT assay). Then, we evaluated mRNA expression and protein expression of membrane metabolite transporters (Gluts and MCTs) and the metabolic enzyme, lactate dehydrogenase (LDH). Moreover, the protein damage, mitochondrial DNA content and mitochondrial protein complexes expression and activity were evaluated to explore the effect of WTEA extract exposure on hSC OS.

III. Material and Methods

1. Chemical

Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12 (DMEM: Ham's F12), Bovine Serum Albumin (BSA), trypsin-EDTA and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal Bovine Serum (FBS) was obtained from Biochrom AG (Germany). NZY M-MuLV Reverse Transcriptase (M-MuLV RT), random hexamer primers, dNTPs, NZTaq 2x Green Master Mix and NZY qPCR Green Master Mix were obtained from NZYTech (Lisboa, Portugal). Primers were obtained from STABVIDA (Oeiras, Portugal). Tween 20 was obtained from AppliChem (Darmstadt, Germany). Dried milk was obtained from Regilait (Saint-Martin-Belle-Roche, France). Antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and Abcam (Cambridge, United Kingdom).

2. Cell Culture

2.1 Primary Culture of Human Sertoli cells

Testicular biopsies were obtained from the Center for Reproductive Genetics Prof.Alberto Barros. Each testicular biopsy was treated as described by Sousa and collaborators (Sousa, Cremades et al. 2002), and hSC primary culture obtained using a routine method (Oliveira, Sousa et al. 2009). In brief, the resulting pellet was suspended in SC culture medium (DMEM: Ham's F12 1:1, pH 7.4, containing 15mM HEPES, 50 U/ml penicillin and 50 mg/ml streptomycin sulphate, 0.5 mg/ml fungizone, 50 ug/ml gentamicin and 10% FBS). Cells were plated on culture flasks and incubated at 37ºC in an atmosphere of 5% CO_2: 95% O_2. The cultures were left undisturbed until day 2, considering the plating day as day 0. Cultures purity was examined by immunoperoxidase detection of Anti-Mullerian hormone and Vimentin specific markers (Steger, Rey et al. 1996). Cells were only used when contaminants below 5% were detected.

2.2 Viability Tests

In order to evaluate the possible cytotoxicity of the two WTEA extracts in study, we performed two viability tests MTT and Sulforhodamine B. The concentration of WTEA were chosen based on published work by our group regarding the effects of a commercial WTEA in rat SCs (Martins, Alves et al. 2014). In that study a concentration of 0.5 mg/mL was used. To our study, besides the concentration 0.5 mg/mL we also found pertinent to test the cytotoxicity of 0.05 mg/mL and 5 mg/mL for the two subtypes of WTEA used (BHYZ and BMD).

MTT Assay

MTT assay, is a colorimetric assay that assesses the ability of viable cells to metabolically reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan crystals. The amount of crystals formed is proportional to the number of viable cells. For this purpose, hSC were seeded in a 24-well plate and allowed to grow until confluence (70-80%). After reaching the desired confluence, the cells were treated for 24h with different WTEA subtype (BHYZ and BMD) and concentrations (0 mg/mL, 0.05 mg/mL, 0.5 mg/mL and 5 mg/mL). At the end of the exposure period, the protocol was performed as described (Martins, Oliveira et al. 2019), and the optical density was measured, using a plate reader (Synergy|H1 Microplate reader, Biotek, Winooski, USA) at 570 nm and 670 nm.

Sulforhodamine B assay

The other test used to evaluate cell cytotoxicity/viability was sulforhodamine B (SRB). This method is based on the ability of SRB dye to stoichiometric bind protein components in acid condition and dissociated in basic conditions. The measured absorbance reflects dye dissociation from the cells, and the dye extracted from cells is directly proportional to cell mass. For this reason, SRB assay is often used to measured cell proliferation. The protocol was performed as previously described (Martins, Oliveira et al. 2019). The optical density was measured at 545 nm using a plate reader (Synergy|H1 Microplate reader, Biotek, Winooski, USA).

3. Experimental Groups

In order to evaluate the effects of WTEA subtypes BHYZ and BMD in hSC, we obtained the cells from six testicular biopsies of men with conserved spermatogenesis. The primary cultures of hSC were allowed to growth until a confluence of 90%.

3.1 Exposure studies to White Tea

After achieving a confluence 90-95% cells were washed with phosphate buffered saline (PBS) and cultured medium replaced by a serum-free medium supplement with DMEM: F12 1:1, pH 7,4. This medium was supplement with insulin-transferrin-selenium (ITS) (10mg/L - 5.5mg/L – 5ug/L, respectively) and no WTEA (control) and 0.5 mg/mL of WTEA, subtype BMD or BHYZ. Cells were maintained for 24 hours, after treatment culture media and SC cells were collected.

3.2 Sertoli cells Culture

To collect the SC, a solution of 10X trypsin-EDTA was used to disassemble the cells from plate. To remove residual trypsin, detached cells were washed with PBS and then centrifugate at 3000g at 4ºC for 6 minutes. The resulting supernatant was discarded, and 1 mL of PBS was added to wash the collected pellet and centrifuged at 5000g on 4ºC for 6 minutes.

4. Total protein extraction and quantification

Sertoli cells were lysed in Radio Immunoprecipitation Assay (RIPAS) Buffer (PBS, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS,100mM of PMSF, supplemented with 100mM sodium orthovanadate and 1% protease inhibitor cocktail). The lysed cells were left 20 minutes on ice and the suspension was then centrifuged at 14000g for 20 minutes at 4ºC. The resulting pellet was discarded. Total protein concentration was quantified using Pierce™ BCA protein Assay Kit (Pierce Biotechnology, Rockford, USA).

5. Extraction of total RNA and synthesis of complementary DNA

Extraction of total SCs RNA was performed using SurePrep™ RNA/DNA/Protein purification Kit (Fisher BioReagents, Porto Salvo, Portugal) following the manufacturer's instructions. RNA concentration and absorbance ratios (260/280) and/or (260/230) were determined by spectrophotometry (NanoDrop 1000, Thermo-Fisher Scientific, Waltham, USA). The RNA obtained from each sample was reversely transcribed using a mixture containing 10 mM of each dNTP, 50 ng of random hexamer primers, 1 µg of RNAt and H_20 sterile until a volume of 13.5 µl and incubated in T100™ Thermal Cycler (Bio-rad, Hemel Hempstead, UK) during 5 minutes to 65ºC. Then 200U NZY M-MuLV Reverse Transcriptase and 2 µl of reaction buffer were added and incubated at 25ºC for 10 minutes, 37ºC for 50 minutes and 70ºC for 15 minutes.

6. Characterization of the oxidative profile

6.1 Extraction of total DNA and quantification of mitochondrial DNA

DNA extraction was performed using SurePrep™ RNA/DNA/Protein purification Kit (Fisher BioReagents, Porto Salvo, Portugal) following the manufacturer's instructions. DNA concentration and absorbance ratios (260/280) and/or (260/230) were determined by spectrophotometry (NanoDrop 1000, Thermo-Fisher Scientific, Waltham, USA). The DNA obtained was used for qPCR to analyse the mitochondrial gene ND1 (Table 2). qPCR was carried

out using a using a CFX Connect™ Real-Time System (Bio-Rad, Hercules, USA). qPCR amplifications used 20 ng of DNA extracted in a 20 µL of final volume reaction containing: 10 µL NZY SYBR qPCR Green Master Mix and 10 µM of forward and reverse primers for each gene.

Amplification conditions comprised an initial denaturation at 95ºC for 5 minutes followed by the 35 cycles of: a) denaturation, 95ºC for 10 seconds; b) annealing at 60ºC for 30 seconds; and c) extension, 72ºC for 1 minute. ß-2-microglobulin (B2M) transcript levels were used to normalize the expression of ND1. The expression of target genes was calculated following the mathematical model proposed by Pfaffl the formula: $2^{-\Delta\Delta Ct}$

Table 2 – Oligonucleotides for amplifications of mitochondrial gene (ND1) and ß-2-Microglobulin (B2M)

Gene	Primer sequence (5'-3')	Amplicon size (bp)
ND1	Forward: CGATTCCGCTACGACCAACT Reverse: AGGTTTGAGGGGGAATGCTG	121
B2M	Forward: GAGGCTATCCAGCGTGAGTC Reverse: GACGCTTATCGACGCCCTAA	306

6.2 Protein Carbonylation

Protein carbonyl content is used as a biomarker for protein oxidation as a result of oxidative damage (Dias, Alves et al. 2015). The amount of protein carbonyl groups in hSC was evaluated using slot-blot technique and a specific antibody. To measure protein carbonyl groups, first the samples were derivatized with 2,4-dinitrophenylhydrazine (DNPH) according to the method developed by Levine and collaborators, (Levine, Garland et al. 1990). In brief, a volume containing 5 ug of protein sample homogenized in phosphate buffer was mixed with same volume of SDS 12% and centrifugated to reduce the nucleic acid interferences. Then the samples were mixed with two volume of DNPH 20mM diluted in trifluoracetic acid (TFA) 10% and incubated for 30 minutes in dark. To stop the reaction, 1.5 volume of Tris 2M diluted in ß-mercaptoethanol 18% was added. Samples were then diluted to a concentration of 0.001 µg/µl using phosphate buffer. A previously activated polyvinylidene difluoride (PVDF) membranes were used in a Hybrid-slot manifold system (Biometra, Göttingen, Germany). The membranes were then blocked during 90 min with 5% non-fat milk in Tris buffered saline solution (TBS) with 0.05% Tween 20%. Subsequently the membranes were incubated overnight with primary anti-DNP in a dilution of 1:5000 (Sigma-Aldrich, St. Louis, MO, USA, D9656). To detect the immune-reactive proteins the membranes were incubated with secondary antibody anti-rabbit IgG-AP in a dilution of 1:5000 (Santa Cruz Biotechnology Heidelberg, Germany, sc 2007) during 90 minutes at room temperature. Membranes were then reacted with Clarity™ Western ECL

substrate (Bio-Rad, Hercules, USA) and the image acquired using a BioRad Fx-Pro-Plus (Bio-Rad, Hemel Hempstead, UK). Densities from each band were quantified using the ImageLab (Vilber Lourmat, Marne-la-Vallée, France). The density of each band was assessed and expressed in fold variation to control.

6.3 Western Blot

Western blot was performed as previously described (Alves, Machado et al. 2011). Briefly, proteins samples (20 µg) were fractionated on 15% SDS-PAGE at 30 mA/gel for 90 minutes and after electrophoresis transferred to Polyvinylidene difluoride (PVDF) membrane at 1.3 A/gel for 7 minutes in a Transblot Turbo apparatus (Bio-Rad, Hercules, USA). The membranes were then blocked in a TBS solution with 0.05% Tween 20 containing 5% of non-fat milk for 90 minutes. Then the membranes were incubated overnight at 4ºC with primary anti-total Oxphos in a dilution of 1:5000 (Abcam, Cambridge, MA, ab110413) or anti-LDH in a dilution of 1:15000 (Abcam, Cambridge, MA, ab52488). The immune-reactive proteins were detected separately with anti-mouse IgG-AP in dilution of 1:10000 (Santa Cruz Biotechnology Heidelberg, Germany, Sc 2008) or anti-rabbit in a dilution of 1:5000 (Santa Cruz Biotechnology Heidelberg, Germany, Sc 2007). Membranes were reacted with and imaged with Clarity™ Western ECL substrate (Bio-Rad, Hercules, USA) and the image obtained using the BioRad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK). The densities from each band were quantified using the ImageLab (Vilber Lourmat, Marne-la-Vallée, France) according to standard methods.

6.4 Mitochondrial complexes Activity

To evaluate the mitochondrial activity it needed to isolate cell mitochondria. The mitochondria fraction was obtained from hSC pellet by differential centrifugation after homogenization with Glass-Teflon potter Elvehjem (Thomas Scientific, New Jersey, USA). Briefly the cell pellet was resuspended in 200 µL of ice buffer (100mM sucrose, 100 mM KCL, 2 mM KH2PO4, 10mM EDTA; pH 7.4) using a glass-Teflon Potter Elvehjem (Thomas Scientific, New Jersey, USA) and centrifugated for 10 min at 1000g, at 4ºC and the supernatant collected. A re-suspension process occurs to improve the isolation, with 100 µL of ice buffer and another centrifugation for 10 min at 1000g, at 4ºC, and the supernatant collected while the pellet is discarded. A supernatant centrifugation was performed for 15 min at 12000g, at 4ºC in order to obtain two fractions a mitochondria-rich fraction (pellet) and mitochondria -free cytosolic fraction (supernatant). The mitochondrial-rich fraction was suspended in 150 µL buffer used.

Activity of complexes I, II, IV and citrate synthase activity in mitochondrial fraction was assessed from absorbance measurements at 37ºC in the microplate reader (Synergy|H1 Microplate reader, Biotek, Winooski,USA), as previously described (Spinazzi, Casarin et al.

2012). Citrate synthase was measured to normalize the complexes activity. Considering that it was done in hSC cell cultures, the protocol was optimized in order to improve the results, whose conditions are summarized in table 3.

Table 3 – Conditions for measure mitochondrial complexes activity and citrate synthase in hSC.

	Complex I	**Complex II**	**Complex IV**	**Citrate Synthase**
λ (nm)	450	600	550	412
Kinetics time (minutes)	20	20	20	10
Buffer	KH_2PO_4 (25 mM) MgCl2 (5mM)	KH_2PO_4 (25 mM)	KH_2PO_4 (25 mM)	Tris-HCL (200 mM) Triton x-100 (0.02%)
pH	7.5	7.5	7.5	8.0
Substrate/ Electron Acceptor	NADH (10 mM) Coenzyme Q (8.89 mM)	Succinate (100 mM) DCPIP (25 mM)	Cytochrome c Reduced (600 µM in buffer)	DTNB (10 mM) Acethyl-CoA (6.1 mM)
Other reagents	KCN (100 mM) Antimycin A (2 mM in ETOH)	KCN (100 mM) Antimycin A (2 mM in ETOH)	Rotenone (500 M in ETOH) Antimycin A (2mM in ETOH)	Oxaloacetate (100 mM)
Inhibitor	Rotenone (500 µM in ETOH)	Oxaloacetate (500 mM)	KCN (100 mM)	_____

7. Characterization of the metabolic profile

7.1 Lactate dehydrogenase Activity

LDH activity was determined using a commercial assay kit - LDH-Cytox (BioLegend, San Diego, CA, Canada) and following the manufacturer's instructions. Briefly, proteins samples (5 µg) were used to determinate the LDH enzymatic activity by measuring absorbance at 490 nm in a microplate reader (Synergy|H1 Microplate reader, Biotek, Winooski,USA). The activity of LDH on samples is proportional to the amount of formazan formed from the conversion of a tetrazolium salt (INT or 3-(4-Iodophenyl)-2-(4-nitrophenyl)-5-phenyl-2H-tetrazol-3-ium chloride). The activities were estimated using the molar absorptivity of formazan and expressed in nmol/min/mg protein.

7.2 Quantitative polymerase chain

The complementary deoxyribonucleic acid (cDNA) was used to analyse monocarboxylate transporter 4 (MCT4), glucose transporter 1 (Glut1) and glucose transporter 3 (Glut3) by qPCR. Specific primers were designed for amplification of the target and housekeeping transcripts (Table 3). qPCR was carried out using a CFX Connect™ Real-Time System (Bio-Rad, Hercules, USA) and efficiency of the amplification was determined for all primers sets using serial dilutions of cDNA, the conditions were optimized, and the specificity of amplicons was determined by melting curves analysis. qPCR amplifications used 1 µL of cDNA (dilution 1:15) in a 16 µL of reaction containing: 8 µL NZY SYBR qPCR Green Master Mix, 5.92 µL H_2O sterile and 10 µM of forward and reverse primers for each gene.

Amplification conditions comprised an initial denaturation at 95 ^{0}C for 5 minutes followed by the cycles of: a) denaturation, 95^{o}C for 10 seconds; b) annealing for 30 seconds (Table 3); and c) extension, 72^{o}C for 1 minute. ß-2-microglobulin (B2M) transcript levels were used to normalize the mRNA expression of MCT4, Glut1 and Glut3. The expression of target genes was calculated following the mathematical model proposed by Pfaffl the formula: $2^{-\Delta\Delta Ct}$

Table 4 - Oligonucleotides and cycling conditions for amplifications of monocarboxylate transporter 4 (MCT4), glucose transporter 1 (Glut1), glucose transporter 3 (Glut3) and ß-2-microglobulin (B2M)

Gene	Primer sequence (5'-3')	Annealing Temperature (ºC)	Amplicon size (bp)	Nº of cycles
MCT4	Forward: TTCGTTTTTGTGCAGGTCCC Reverse: GTCAGTCCCATCCCAGAACG	55	268	40
Glut1	Forward: AGCAGCAAGAAGCTGACGGGTC Reverse: CGCCGGCCAAAGCGGTTAAC	58	269	40
Glut3	Forward: TCAGGCTCCACCCTTTGCGGA Reverse: TGGGGTGACCTTCTGTGTCCCC	54	230	35
B2M	Forward: ATGAGTATGCCTGCCGTGTG Reverse: CAAACCTCCATGATGCTGCTTAC	58	90	40

7.3 Proton nuclear magnetic resonance spectroscopy

Proton nuclear magnetic resonance (^{1}H-NMR) spectra of hSC extracellular culture media was acquired and quantified as previously described by our team (Alves, Oliveira et al. 2011, Alves, Neuhaus-Oliveira et al. 2013). Sodium fumarate was used was as an internal reference (6.50 ppm, singlet) to quantify the metabolites present in hSC extracellular media (ppm,

multiplet) : lactate (1.33, doublet); alanine (1.45, doublet); acetate (1.90, singlet); pyruvate (2.35, singlet) and H1-α-glucose (5.22, doublet). Relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro NMR spectral analysis program.

8.Statistical analysis

The statistical significance of experimental was assessed by one-way ANOVA followed by multiple comparisons through Fisher Least Significant Difference (LSD) post-test. All experiments were performed in triplicated and results presented as mean ± SEM (n= 6 for each condition) with $p < 0.05$ considered significant. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, USA).

IV. Results

1. The WTEA subtype BMD extract induces cell cytotoxicity at concentration of 5 mg/mL in hSC

The cytotoxicity profile of WTEA extracts subtypes BHYZ and BMD was characterized through *in vitro* assay. Cells were incubated for 24h with different WTEA subtypes (BHYZ and BMD) and concentrations (in mg/mL: 0– control, 0.05, 0.5 and 5). The MTT assay was performed to analyse cellular viability through the study of the cells metabolic activity of (Figure 5).

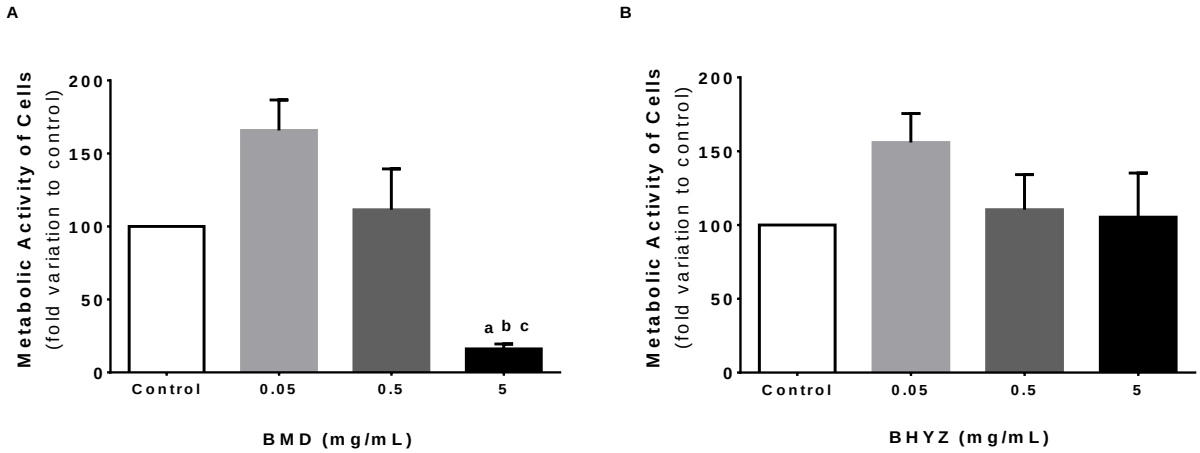


Figure 5 - Evaluation of the metabolic activity of hSC after 24h incubation with increase concentration of white tea subtypes extract BMD and BHYZ as determined by MTT assay. The results attain for tea subtypes BMD (Panel A) and BHYZ (Panel B) are presented. Results are expressed fold variation to control and mean ± SEM (n=3, for each condition). Significantly different results ($p < 0.05$) are indicated as: a) relative to control; b) relative to 0.05 mg/mL; c) relative to 0.5 mg/mL.

This assay shows that the metabolic activity of the cells is not affected after exposure to the lowest concentration of WTEA extract subtype BMD but at the concentration of 5 mg/mL there is a severe reduction of the cellular metabolic activity (16 ± 3-fold variation to control) thus showing that at this concentration, the exposure to the subtype BMD extract is very deleterious for hSC (Figure 5, panel A). In the other hand, exposure to the WTEA subtype BHYZ presented no significant alterations (Figure 5, panel B).

In order to asses if 24h exposure to the different extracts of WTEA subtypes can affect the growth of hSCs, the SRB assay was performed. We observed that exposure to the extract of WTEA at 0.05 mg/mL, for both subtypes BMD (Figure 6, panel A) and BHYZ (figure 6, panel B), induces a significant cellular growth when compared with the control (225 ± 43-fold variation to control after exposure to subtype BMD and 238 ± 44-fold variation to control after exposure to BHYZ subtype). However, to the other studied concentrations, for the extracts of both WTEA subtypes, there was no significant alterations in cell growth when compared with the control. However, there was a significant reduction of cellular growth after exposure to the extracts of both subtypes, BMD and BHYZ, when compared to the concentration of 0.05 mg/mL (Figure 6, Panel A and B). Notably, these results suggest that cellular growth is stimulated when WTEA

extracts, subtypes BMD and BHYZ, are presented but the highest concentrations have no effect (positive or negative) in cellular growth. Thus, regarding these last results, we selected the concentration of 0.5 mg/mL for both subtypes, for comparative purposes and also to study some molecular mechanisms by which WTEA extract, for the different subtypes, can act in hSC.

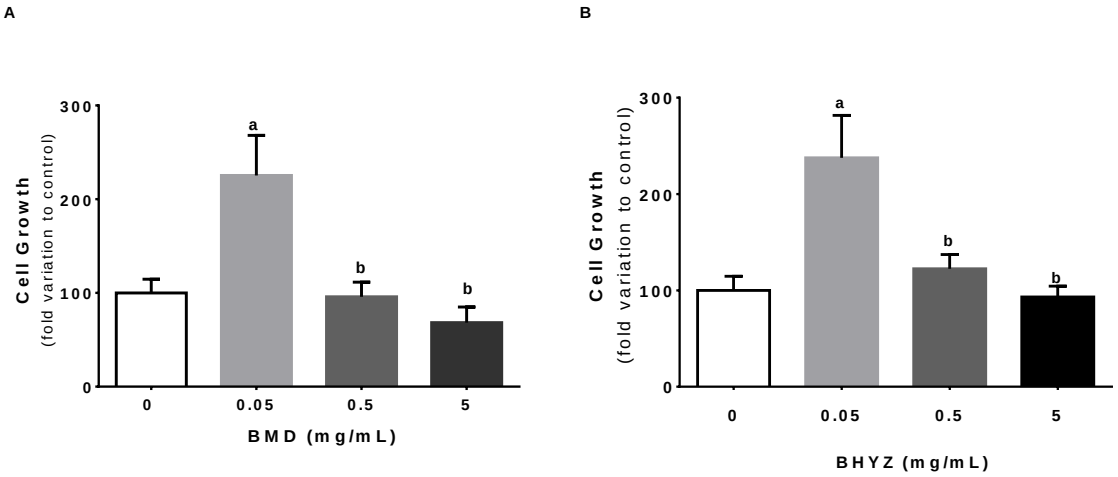


Figure 6 - Cellular growth of hSC after 24h incubation with increasing concentrations of WTEA extract of subtypes BMD and BHYZ as studied by SRB assay. The results attained for tea subtypes BMD (Panel A) and BHYZ (Panel B) are presented. Results are expressed fold variation to control and mean ± SEM (n=3, for each condition). Significantly different results ($p < 0.05$) are indicated as: a) relative to control; b) relative to 0.05 mg/mL.

2. Exposure to WTEA extract of both subtypes enhance pyruvate consumption and acetate production but promote a distinct lactate/alanine ratio

Glucose is the preferred substrate of hSC. After the import to SCs, glucose is metabolized via glycolysis, resulting in ATP and pyruvate. Our results demonstrate that the levels of glucose consumption in hSC exposed to WTEA extract of both subtypes (0.5 mg/mL) remain unchanged when compared with the control (Figure 7, Panel A). Interestingly, the consumption of, pyruvate is increased in cells exposed to tea WTEA extract of subtypes BHYZ and BMD to 0.62 ± 0.11 and 0.59 ± 0.11, respectively when compared with the control cells that presented a pyruvate consumption of 0.20 ± 0.08 (Figure 7, Panel B).

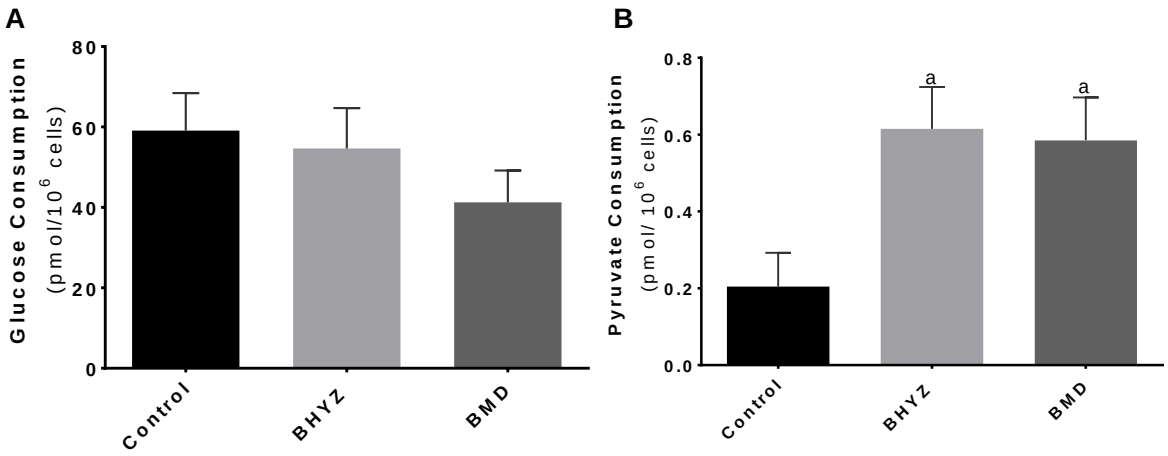


Figure 7 – Effect of 24h hSC exposure to WTEA extract of subtypes BHYZ and BMD glucose and pyruvate consumption. Glucose consumption (Panel A) and pyruvate consumption (Panel B) in hSC non exposed (control) or exposed to two different subtypes of WTEA: BHYZ and BMD. Results are expressed fold variation to control and mean ± SEM (n=6, for each condition). Significantly different results ($p < 0.05$) are indicated as: a) relative to control.

Pyruvate obtained in the end of glycolysis pathway can be metabolized in three different ways a) originate lactate, b) be metabolized to alanine or c) be transported to the matrix of mitochondria and form acetyl-CoA. All these pathways are important, but lactate production is essential as this metabolite is necessary for the correct functioning of spermatogenesis because the developing germ cells use this metabolite as energy source. Our results show the exposure of hSC to any of the WTEA subtypes extract does not change the production of this essential metabolite for germ cells (Figure 8, Panel C).

Regarding alanine our results showed that cells exposed to the WTEA extract BMD presented a significant reduction in the production of alanine (0.11 ± 0.03) relative to non-exposed cells (0.35 ± 0.08), while in cells exposed to the WTEA subtype BHYZ there was a consumption of alanine (-0.05 ± 0.02) (Figure 8, Panel A). The presence of acetate in SC has been overlooked but there is a great interest as this metabolite is the most common intermediate for synthesis of fatty acids or cholesterol (Alves, Socorro et al. 2012), coming from acetyl-CoA. Our results show that there was a production of acetate in hSC cells exposed to both WTEA subtypes whereas non-exposed cells demonstrated a small consumption of this metabolite (Figure 8, Panel B). The lactate/alanine ratio reflects the redox state of the cell, once it is related to the balance between reduced NADH/NAD+ oxidized. Our results demonstrate that hSC exposed to the WTEA extract of BHYZ present a lactate/alanine ratio of - 46.33 ± 22.18 while hSC exposed to BMD presented a ratio of 91.76 ± 28.07 (Figure 8, Panel D).

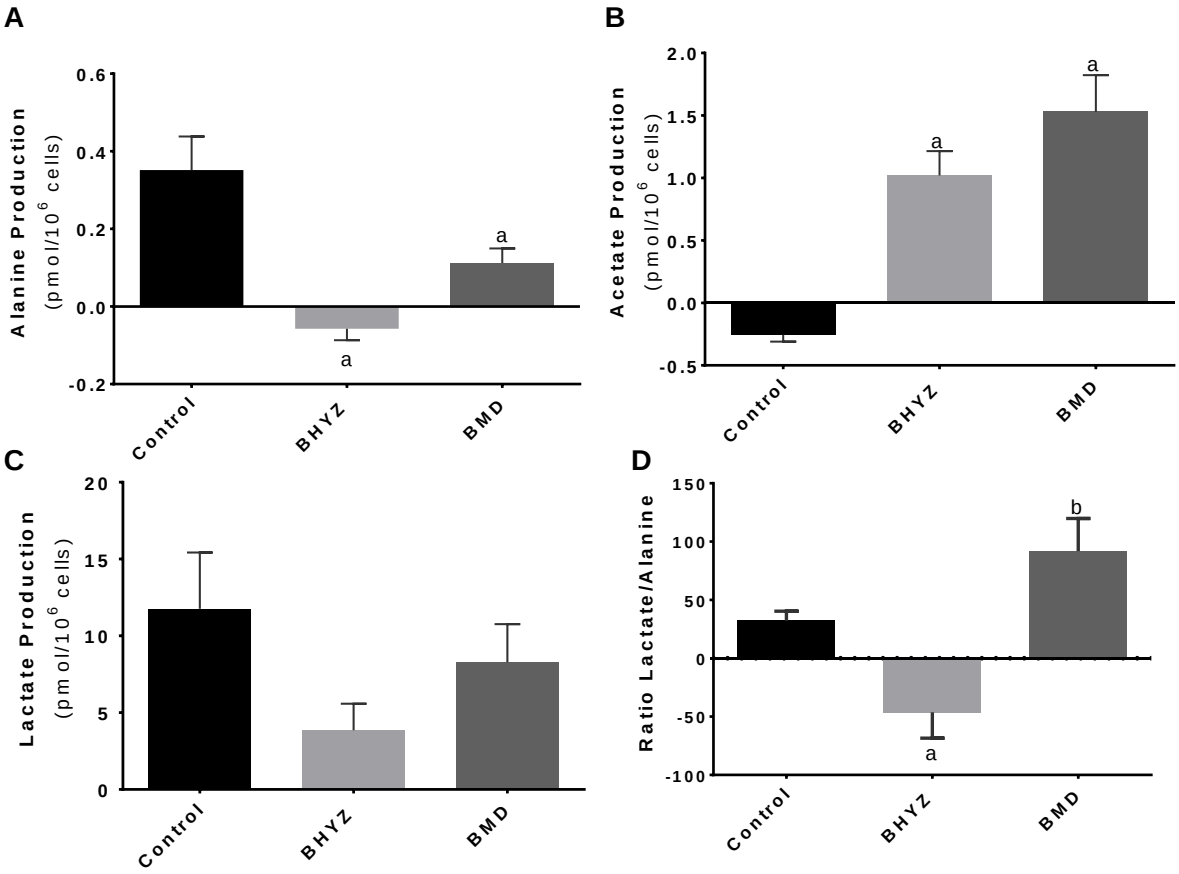


Figure 8 - Effect of 24h hSC exposure to WTEA extract of subtypes BHYZ and BMD in the production of alanine, acetate and lactate, and ratio lactate/alanine. Alanine production (Panel A), acetate production (Panel B), lactate production (Panel C) and ratio Lactate/Alanine (Panel D) in hSC non exposed (Control) or exposed to two different subtypes of WTEA: BHYZ and BMD. Results are expressed fold variation to control and mean ± SEM (n=6, for each condition). Significantly different results ($p < 0.05$) are indicated as: a) relative to control, b) relative to WTEA extract subtype BHYZ.

3. Exposure to the extract of both WTEA subtypes modulates glycolysis related transporters and decreases lactate dehydrogenase activity

In hSC, like in almost all cell types, glucose intake is secured by the function of glucose transporters (Gluts), being that in these cells Glut 1 and Glut 3 are the most relevant transporters ans thus, we evaluate their mRNA expression in hSC, of our experimental groups. Our results show a significant increase in both transporters in cells exposed to both WTEA subtypes (6 ± 0.2 for BHYZ tea and 5 ± 1 for BMD tea) when compared to non-exposed cells to (1 ± 0.4) (Figure 9, Panel A and B).

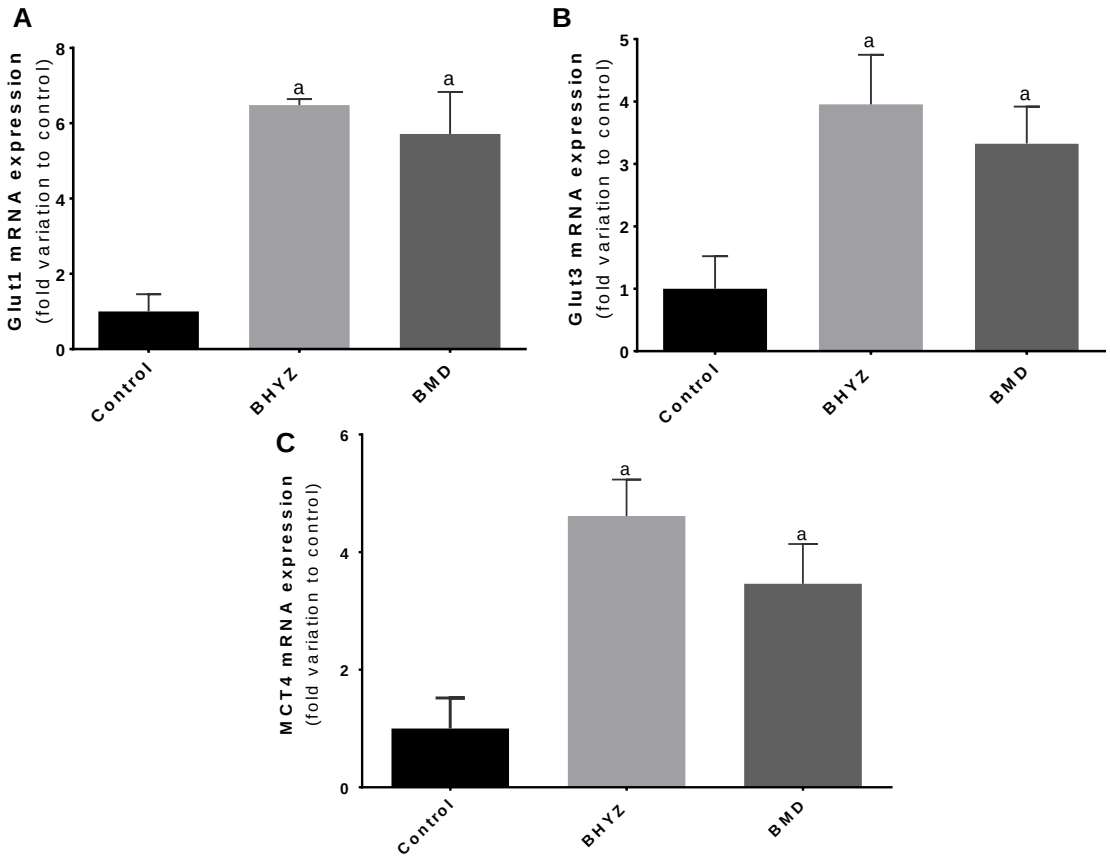


Figure 9 – Effect of 24h hSC exposure to WTEA extracts of subtypes BHYZ and BMD in mRNA expression of glucose transporters and monocarboxylate transporter. Glucose transporter 1 (Glut 1) (Panel A), glucose transporter 3 (Glut 3) (Panel B) and monocarboxylate transporter 4 (MCT4) (Panel C) in hSC non exposed (Control) or exposed to two different subtypes of WTEA: BHYZ and BMD. Results are expressed fold variation to control and mean ± SEM (n=6, for each condition). Significantly different results ($p < 0.05$) are indicated as: a) relative to control.

Monocarboxylate transporter 4 (MCT 4) presence and function in hSC has an important role for spermatogenesis as it is the main responsible for the export of lactate to the intratubular fluid. The export of lactate from hSC to the intratubular fluid allows developing germ cells to have access to this metabolite that is the main energetic substrate for those cells. Thus, we assessed the mRNA expression of MCT4. Our results show that, there is a significant increase of this transporter in hSC exposed WTEA extract of the subtypes BHYZ and BMD to 4.6 ± 0.62 and 3.5 ± 0.68, respectively when compared with non-exposed cells (control - 1 ± 0.5) (Figure 9, Panel C).

The production of lactate from pyruvate is mediated by the lactate dehydrogenase (LDH). Thus, we evaluated the protein expression of LDH and detected a significant decrease in its expression in cells exposed to the extract of WTEA subtype BHYZ to 0.20 ± 0.09 when compared with non-exposed cells that presented as expression of 1 ± 0.2 (Figure 10, Panel A). In cells exposed to the WTEA extract subtype BMD, there was a non-significant decreased in LDH (0.56 ± 0.2 – fold variation to control) when compared with the non-exposed cells (Figure

10, Panel A). To further characterize these mechanisms, we evaluated the LDH activity, in order to verify if the results would corroborate with the data obtained regarding the protein expression. Indeed, hSC exposed to the WTEA extract of subtypes BHYZ and BMD showed a significant reduction of LDH activity to 101 ± 10 and 146 ± 19, respectively, when compared with non-exposed cells (control) which presented an activity of 390 ± 40 (Figure 10, Panel B).

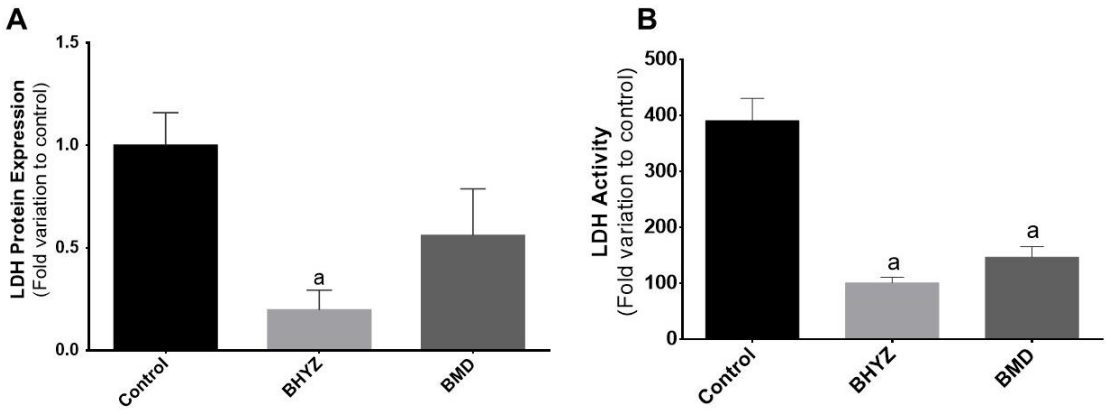


Figure 10 – Effect of 24h hSC exposure to WTEA extracts of subtypes BHYZ and BMD in lactate dehydrogenase (LDH) protein expression and activity. LDH protein expression (Panel A) and activity (Panel B) in hSC non exposed (control) or exposed to two different subtypes of WTEA: BHYZ and BMD. Results are expressed fold variation to control and mean ± SEM (n=6, for each condition). Significantly different results ($p < 0.05$) are indicated as: a) relative to control.

4. Exposure to WTEA extract subtypes BHYZ and BMD decreases the expression of mitochondrial complex V

Mitochondria are very important organelles for several physiological functions, particularly to cellular respiration, since their inner membrane contains the respiratory chain. The respiratory chain present in mitochondrial membranes consists of five multimeric protein complexes that function based on electron transport producing energy for cell. Thus, we evaluate the protein expression of the mitochondrial complexes. We observed that exposure of hSC to WTEA extract subtype BMD significantly increased complex I protein levels to 2.9 ± 0.9 -fold variation to control when compared with non-exposed hSC (Figure 11). However, the expression of mitochondrial complex V was significantly decreased after exposure to the WTEA extract of both subtypes BHYZ and BMD to 0.22 ±0.12 and 0.50 ± 0.16, respectively, when compared with non-exposed cells which presented an expression of 1 ± 0.09 (Figure 11).

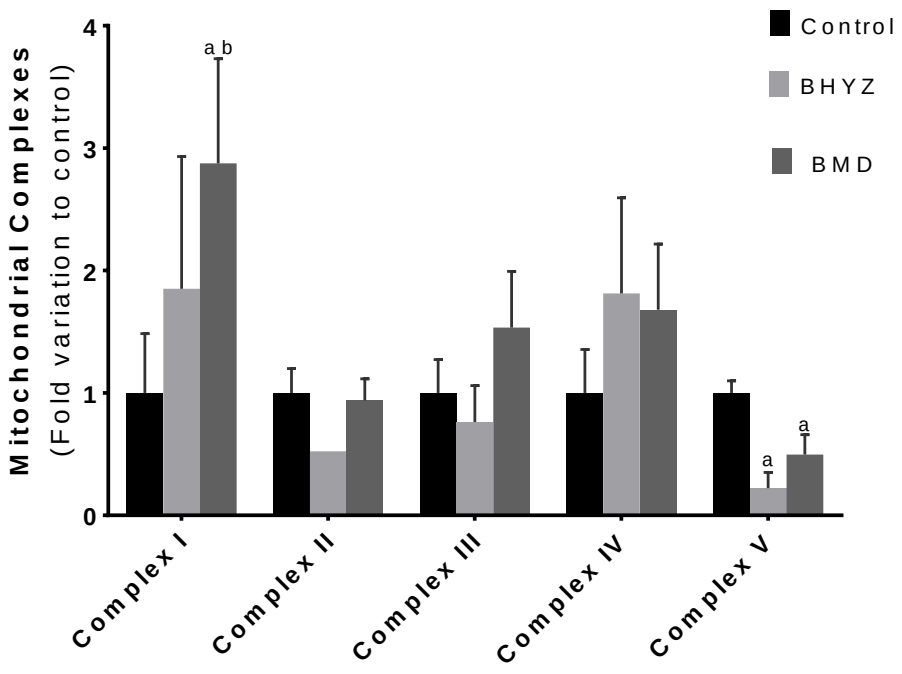


Figure 11 – Effect of 24h hSC exposure to WTEA extracts of subtypes BHYZ and BMD in mitochondrial complexes protein expression. Mitochondrial complexes expression in hSC non exposed (control) or exposed to two different subtypes of WTEA: BHYZ and BMD. Results are expressed fold variation to control and mean ± SEM (n=6, for each condition). Significant different results ($p < 0.05$) are indicated as: a) relative to control, b) relative to WTEA extract subtype BHYZ.

The mitochondrial activity of complexes I, II and IV was also measured (Figure 12), and the results show a significant increase in the activity of complex II in hSC exposed to WTEA extract subtype BMD (to 1.09 ± 0.22 – fold variation to control) when compared with non-exposed cells (Figure 12, Panel C).

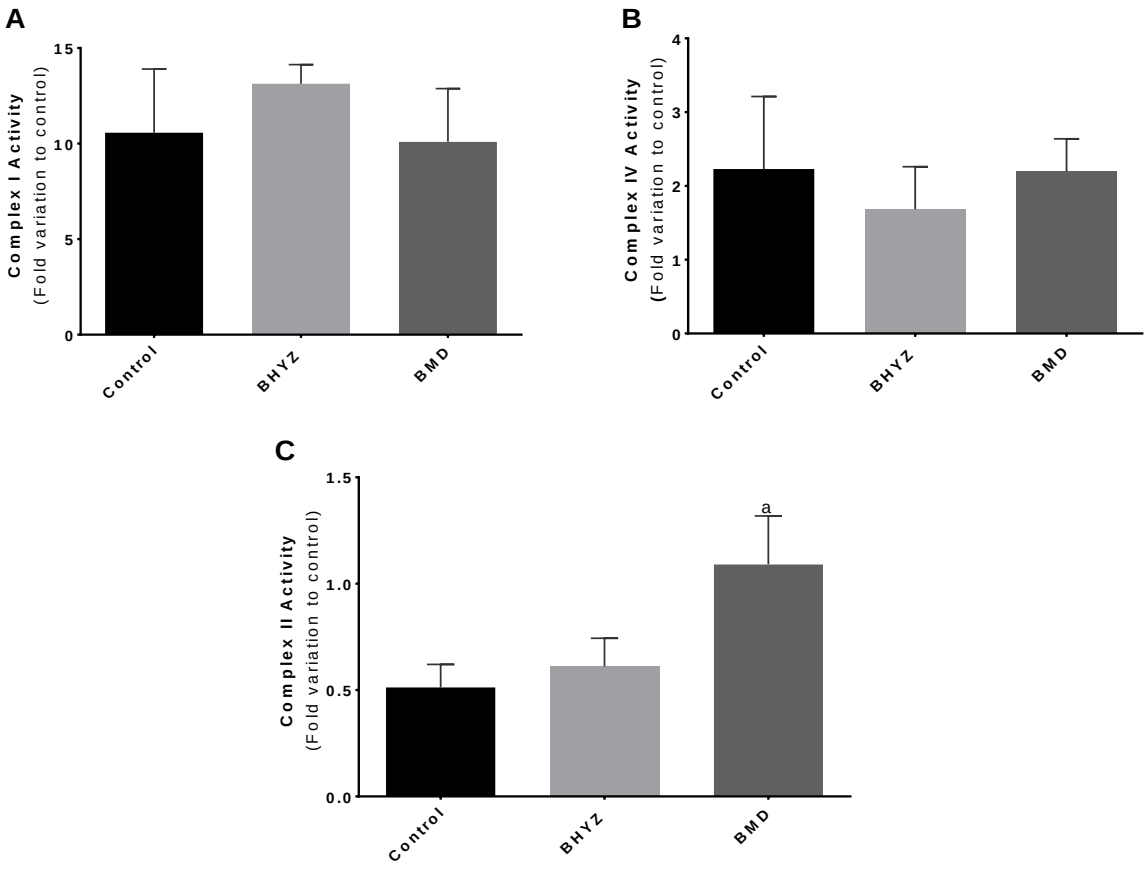


Figure 12 – Effect of 24h hSC exposure to WTEA extracts of subtypes BHYZ and BMD in mitochondrial complexes activity. Complex I (Panel A), complex IV (Panel B), Complex II (Panel C) activity in hSC non exposed (control) or exposed to two different subtypes of WTEA: BHYZ and BMD. Results are expressed fold variation to control and mean ± SEM (n=5, for each condition). Significant different results ($p < 0.05$) are indicated as: a) relative to control.

5. Exposure to WTEA extract subtypes BHYZ and BMD decreases protein carbonylation and mitochondrial DNA content is differentially affected

The respiratory chain is one of the main sources of reactive oxygen species (ROS), and their excessive production leads to a cellular imbalance resulting in reduced antioxidant defences and thus, culminating in cellular damage. Carbonylation is a biomarker for protein oxidative damage caused in situations of oxidative stress. As a tea extract is rich in phytochemicals with antioxidant properties, we measured protein carbonylation. Our results show that hSC exposure to WTEA extract of both subtypes, BHYZ and BMD, significantly decreased to 0.32 ± 0.05 and 0.20 ± 0.06, respectively when compared with non-exposed cells which presented an proteins carbonylation levels at 1.5 ± 0.25 (Figure 13).

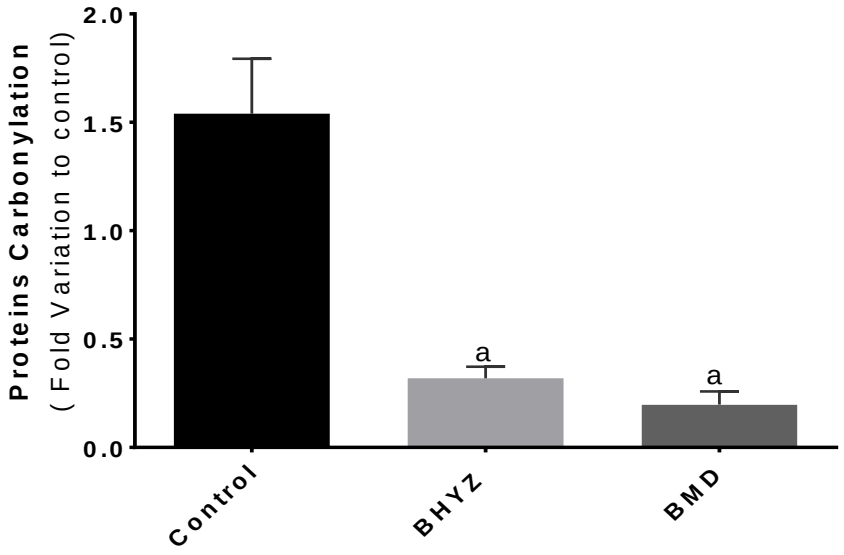


Figure 13 – Effect of 24h hSC exposure to WTEA extracts of subtypes BHYZ and BMD in protein carbonylation. Results are expressed fold variation to control and mean ± SEM (n=6, for each condition). Significant different results ($p < 0.05$) are indicated as: a) relative to control.

Alteration in oxidative homeostasis may lead to changes in mitochondrial DNA content. Therefore, we evaluated if exposure of hSC of any the WTEA extract subtypes can alter the mitochondrial content. Our results show that hSC exposure to WTEA extract subtype BHYZ increase mitochondrial DNA content (1.23 ± 0.19) when compared to that detect in hSC exposed to WTEA extract subtype BMD (0.71 ± 0.17), though it is not significantly different to the mitochondrial DNA content in non-exposed cells (1 ± 0.11)(Figure 14).

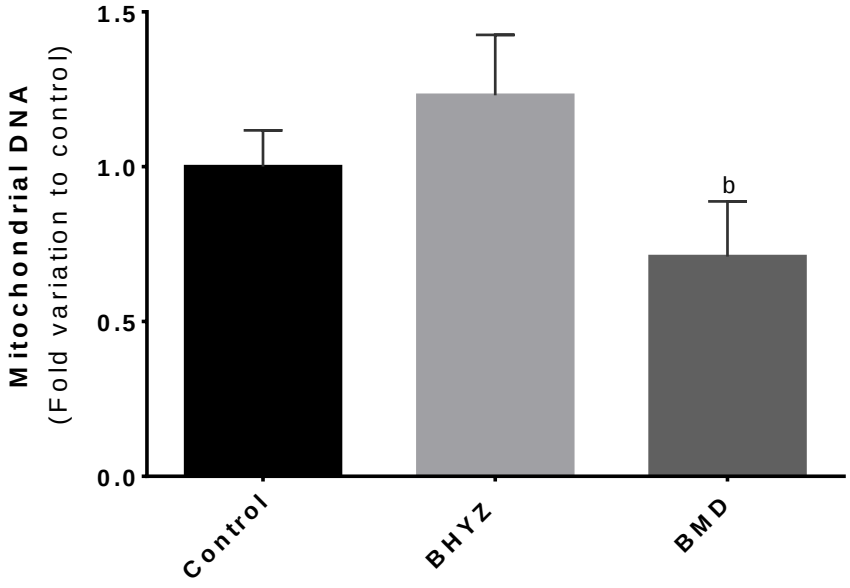


Figure 14 – Effect of 24h hSC exposure to WTEA extracts of subtypes BHYZ and BMD in mitochondrial DNA content. Results are expressed fold variation to control and mean ± SEM (n=6, for each condition). Significant different results ($p < 0.05$) are indicated as: a) relative to control, b) relative to WTEA extract subtype BHYZ.

V. Discussion

Tea (*C. sinensis*) is the second most consumed beverage in the world, and has been reported to have several health promoting benefits (Dias, Tomás et al. 2013). Many of those benefits have been attributed to the bioactive components present in tea which are considered natural antioxidants. It is known that the antioxidant capacity of tea is due to its high content of polyphenols, which in white tea, are found in leaves and constitute around 30% of dry weight (Dias, Tomás et al. 2013). The content of phytochemical components such as, polyphenols, caffeine, gallic acid and others is reported to be higher in white tea than in green tea (Hilal and Engelhardt 2007). Indeed, a previous study from our group characterized the phytochemical composition of commercial white and green tea and showed that the former has a higher concentration of polyphenols when compared to the latter (Dias, Alves et al. 2014), particularly due to its higher concentration of catechins. In addition, it was suggested that the antioxidant activity of white tea is due to its high content in these phytochemical components that possess beneficial properties. Several studies show that the catechins present in WTEA are powerful antioxidants, with ROS scavenging capacity (Nakagawa and Yokozawa 2002) and metal chelators (Soobrattee, Neergheen et al. 2005). Other tea constituents also have beneficial and interesting effects on human health already described, such as caffeine and L-theanine (Martins, Alves et al. 2014). L-theanine has been reported as a relaxing agents (Rogers, Smith et al. 2008, Cooper 2012) also with anti-diabetic and memory loss preventive properties(Zheng, Sayama et al. 2004, Matsumoto, Yamamoto et al. 2005, Kim, Lee et al. 2009). Caffeine is also reported to have several health benefits, including weight loss (Cano-Marquina, Tarin et al. 2013) and has neuroprotective properties (Duarte, Carvalho et al. 2009). Although some studies are focused on the consumption of white tea, its mechanisms of action are still unknown. Spermatogenesis depends of a well controlled metabolic and oxidative homeostasis, particularly in SC, which are responsible for the nutritional and physical support of the developing germ cells (Alves, Rato et al. 2013). On one hand, the metabolic cooperation between SC and developing germ cells is essential due to the dependence of lactate by the latter though it may be associated with ROS production. On the other hand, excessive levels of ROS contributed to a reducing antioxidant activity of SC, causing dysfunctional spermatogenesis and consequently subfertility or infertility in men (Tremellen 2008, Dada and Bisht 2017). So, in this work we proposed to study the effects of the exposure to two subtypes of white tea (BHYZ and BMD) on hSC function.

Firstly, we had to select some concentration of WTEA extract of both subtypes BHYZ and BMD. We selected the concentration (in mg/mL): 0.05; 0.5 and 5 based on previous studies performed by our group (Dias, Alves et al. 2014, Alves, Martins et al. 2015). This first assessment showed that the WTEA subtype BMD extract induces cytotoxicity to hSC exposed at the highest concentration. The same concentration did not change the metabolic activity or cell growth when hSC were exposed to the WTEA extract subtype BHYZ. These results suggest that at higher concentration, the use of this WTEA extract may be deleterious, at least for hSC. Nevertheless, further studies will be needed. For our further studies that aimed compare both WTEA extract subtypes and their mechanism of action, we selected the concentration of 0.5 mg/mL since our results showed that at this concentration none of the subtypes of extracts induce cytotoxicity

in hSC. In addition, our group has done a previous study in rat SC with this concentration (Martins, Alves et al. 2014) thus allowing us to compare how commercial WTEA extract differs from a genuine WTEA subtype BMD and BHYZ while compare effects in rat and hSC.

In this work we exposed hSC to extracts of two subtypes of WTEA, BHYZ and BMD, which are very similar being that the main difference between them is related with the leaves selected for their production (Damiani, Bacchetti et al. 2014, Ning, Ding et al. 2016, Lin, Xia et al. 2017). BHYZ is the tea produced with parts of the plant that are under initial development, as it is produced only by macerating the leaves buds, while BMD is also produced with the buds, but immature leaves are added. It is not clear which of these two subtypes of WTEA has a higher content in phytochemical components, which trigger beneficial effects on human health, but some factors influence its content, such as conditions of plant growth, geographical origin and processing (de Mejia, Ramirez-Mares et al. 2009).

Sertoli cells are essential for the success of spermatogenesis in such a way that these cells are reported to adapt their metabolism when extreme situations in non physiological situations, to sustain lactate production (Alves, Rato et al. 2013). However, when they are in the presence of excessive amount of toxic agents and their defences become reduced, their adaptation is limited and thus, deleterious effects can occur (Alves, Neuhaus-Oliveira et al. 2013). Taken in consideration that metabolic and oxidative profile are very similar and that WTEA was already reported as a metabolic modulator of rat SC, we focuses our work on the effects of the different WTEA subtypes (BHYZ and BMD) extracts on the metabolic and oxidative profile of SC. Sertoli cells exhibit a Warburg-like metabolism, i.e. they function metabolically in a very similar way to cancer cells (Oliveira, Martins et al. 2015). Indeed, these cells use glucose as their main energy source, choosing predominantly the glycolysis. The entrance of glucose from the extracellular medium into the SC is controlled by the expression of glucose transporters, mainly Glut 1 and Glut 3 (Angulo, Rauch et al. 1998, Riera, Galardo et al. 2009, Alves, Rato et al. 2012, Oliveira, Alves et al. 2012). These hSC exposed to WTEA subtypes (BHYZ and BMD) extracts had a significantly higher expression of Glut 1 and Glut 3 mRNA relative to the non-exposed hSC, thus suggesting an increased glycolytic flow, though glucose consumption remains unchanged. Studies report the that high metabolic adaptability of hSCs to external conditions occurs through a modulation of Gluts expression as happens in the absence of insulin or glucose (Riera, Galardo et al. 2009, Oliveira, Alves et al. 2012). Therefore, we propose that this increased expression of Gluts after the exposure to WTEA extracts subtypes BHYZ and BMD is an adaptation of hSC to sustain glucose consumption and not compromised the glycolytic flux.

Inside the cells, glucose is then metabolized via glycolysis, originating pyruvate that can be processed in three different pathways and consequently give rise to different metabolites essential to perform other metabolic pathways and to the metabolic cooperation that occurs in the testis (Gray, Tompkins et al. 2014, Martins, Alves et al. 2014, Oliveira, Martins et al. 2015). Cells exposed to WTEA extract presented a significantly higher consumption of pyruvate, further supports that glycolytic flux changed in exposed cells. One of the metabolites

that the consumption of pyruvate can originate is lactate. Lactate is an essential metabolite for developing germ cells, as it is their main source of energy; stimulates the synthesis of RNA and proteins in spermatids and allows the survival of these cells (Jutte, Jansen et al. 1982, Boussouar and Benahmed 2004)). Lactate is exported from hSC by specific transporters, the monocarboxylate transporter 4 (MCT4) (Boussouar and Benahmed 2004, Oliveira, Martins et al. 2015). Our results show that exposure of hSC to WTEA extracts BHYZ and BMD presented an increase in the mRNA expression of MCT4 supporting an effect on the export lactate or acetate. The reversible reaction that converts pyruvate to lactate is catalysed by LDH. Thus, we analysed the protein expression and activity of LDH. Our results show that hSC exposed to WTEA extract of both subtypes BHYZ and BMD had significantly lower activity of LDH though the expression was only decreased after hSC exposure to WTEA extract subtype BHYZ. These results were followed by sustained lactate production after exposure to both WTEA extracts subtypes BHYZ and BMD. In sum, exposure to WTEA extract subtype BHYZ and BMD stimulates the expression of glycolysis-related transporters, particularly Gluts and MCT4, while also decreasing LDH enzyme activity, and maintaining lactate production. Thus, we propose that exposure to these WTEA extract leads to metabolic adaptation that allow hSC to sustain lactate production even with lower LDH activity. These mechanisms can be very relevant under conditions that comprise lactate production by hSC such as those that occur in metabolic diseases. Further studies will be needed to prove this hypothesis.

The final glycolysis product, pyruvate, can be metabolised by a pathway that originates alanine through the action of aminotransaminase (Yang, Blaileanu et al. 2002). Our results showed that hSC exposed to WTEA extract subtype BHYZ consume alanine while hSC exposed to WTEA subtype BMD produce alanine. Metabolically, alanine serves only as a source of pyruvate which is subsequently used in other metabolic pathways such as the krebs cycle or gluconeogenesis (Rato, Alves et al. 2012) but it also can be incorporated. But importantly, alanine regulation is essential for redox homeostasis. Indeed, lactate/alanine is often regarded as index for cellular redox state (O'Donnell, Kudej et al. 2004) thus reflecting the balance between NADH/NAD^+. As hsc exposed to WTEA extract subtype BHYZ consumed alanine, they have a much lower ratio than the non-exposed cells, whereas cells exposed to WTEA extract subtype BMD tea have a significantly higher ratio than cells exposed to WTEA extract subtype BHYZ tea. This clearly sets difference between the two subtypes of WTEA regarding the metabolism induced redox state. It appears that WTEA extract subtype BMD is capable to increase the metabolic production of relevant intermediates by hSC though lowering oxidative stress, as demonstrate by the lower lactate/alanine ratio.

Finally, pyruvate can be converted in the mitochondrial matrix to acetyl-CoA (Alves, Socorro et al. 2012). The hydrolysis of acetyl-CoA through acetyl-CoA hydrolase originates acetate (Yamashita, Itsuki et al. 2006) that is a pivotal metabolite since it is one of the main sources of fatty acids and cholesterol (Yoshimoto, Waki et al. 2001). The acetate its known as only carbon source metabolite used for de novo lipids synthesis (Howard 1977) of developing

germ cells (Alves, Socorro et al. 2012, Martins, Moreira et al. 2015). A previous study from our group showed that hSC in culture produce high amount of acetate and proposed that is may be associated to the constant need of cholesterol and other sources for the synthesis of new developing germ cells (Alves, Socorro et al. 2012). Our study shows that both WTEA extract subtypes stimulate hSC acetate production. This significant increase in acetate production and its extraction from mitochondria may be related to the role of this metabolite in the development of germ cells and where it acts as a central regulator of spermatogenesis once it is the energy source for the synthesis of new lipids essential for germ cells division and formation (Howard 1977, Alves, Socorro et al. 2012, Martins, Moreira et al. 2015). However, further studies are needed to clarify the role of acetate in the development and division of germ cells. Taken together, our results show that exposure hSC to both WTEA extracts maintains lactate production via glycolysis but nonetheless stimulates energy production through the krebs cycle, since much of the pyruvate appears to go to mitochondria and acetate production is also stimulated.

Mitochondria contains its own DNA (mtDNA) and is very important due to its energy production capacity allowing cellular respiration (Folgerø, Bertheussen et al. 1993, Sousa, D'Imprima et al. 2018). In its internal membrane there are the mitochondrial complexes also called OXPHOS, which generate a driving force that pumps protons out of the mitochondrial matrix and thus stimulates the synthesis of ATP through the transport of electrons (Venkatesh, Deecaraman et al. 2009). The five complexes found in the internal membrane of the mitochondria constitute the chain of electron transference or respiratory chain. However, only the complexes I, III and IV pump protons to the outside of the membrane (Folgerø, Bertheussen et al. 1993). The exposure of hSC to WTEA extract of both subtypes BHYZ and BMD alters the protein expression of these complexes, particularly of complex V, whose expression was significantly decreased. However, exposure of hSC to WTEA extract subtype BMD significantly increased complex I expression and complex II activity. The complexes I and II are electrons acceptors where electron acceptance in complex I occurs by catabolic reactions in the mitochondrial matrix, while the complex II is part of the Krebs cycle and thus, the electrons derived from succinate (Dudkina, Sunderhaus et al. 2008). The complex V also called ATP synthase which forms ATP through ADP and Pi due to the energy released when the protons pass back into the mitochondrial matrix (Schultz and Chan 2001). Our results show that hSC exposure to WTEA extract subtype BMD stimulates the protein expression of mitochondrial complex I though not stimulating its activity which ensures the complex functioning without increasing the levels of protons pumping outwards, which can be harmful because it can cause reactive oxygen species leak that then will be harmful, and complex I is the complex that mostly produces reactive oxygen species (Sousa, D'Imprima et al. 2018). Nevertheless, the exposure of hSC to WTEA extract subtype BMD increases the activity of the complex II which can be positive as it sustains the respiratory chain without pumping protons outwards and also participates in the Krebs cycle. The decrease of mitochondrial complex V after exposure to both WTEA extract subtypes BHYZ and BMD, suggests that cellular respiration can be maintained at normal levels

without need the normal expression of this complex. This is positive since mitochondrial complex V acts with the entry of protons, which can become harmful to the cell since some studies show a loss of protons (proton leak) consequently producing ROS induced damage to the cells (Cheng, Nanayakkara et al. 2017). The respiratory chain is the main source of ROS production that at excessive levels can be harmful to the cell causing, among other things, damage in proteins, RNA or even in DNA. Mitochondrial complexes are not fully encoded by mtDNA since there are complexes that are encoded by mtDNA and genomic DNA (Folgerø, Bertheussen et al. 1993, Venkatesh, Deecaraman et al. 2009). Therefore, mutations in the genomic DNA or mtDNA may condition the correct functioning of the respiratory chain and consequently interfere in the production of ATP (Venkatesh, Deecaraman et al. 2009). Our results show that there are no changes in the number of mitochondrial DNA copies of hSC exposed to WTEA extract subtypes BHYZ and BMD when compared to non-exposed cells. However, when we compare the both WTEA extract subtypes, our data shows that hSC exposed to WTEA extract subtype BMD induces a decrease in the mitochondrial DNA content when compared with the mitochondrial DNA content detected in hSC exposed to WTEA extract subtype BHYZ. This illustrates that BMD subtype promotes a decrease on hSC mitochondrial DNA that is followed by increases in mitochondrial complex I expression and mitochondrial complex II activity. Further studies are needed to access if these events are interlinked and are cause or consequence. In addition, these changes in mitochondrial physiology and functioning are usually linked to ROS overproduction and oxidative stress. Nevertheless, WTEA extract is known to have a great antioxidant, thus counteracting ROS and reducing damage induced by those ROS. Protein carbonylation is a biomarker for OS, and hSC exposed to WTEA extract both subtypes BHYZ and BMD show a significant reduction in carbonylation which corroborates with other studies from our group (Alves, Martins et al. 2015, Nunes, Alves et al. 2015, Oliveira, Tomás et al. 2015) showing that the antioxidant properties of WTEA make it very useful for situations of OS.

VI. Conclusions

Tea is a beverage greatly consumed worldwide, only surpassed by water. Although its interesting health promoting properties have been described for centuries, the mechanisms of action remain unknown. White tea is the less studied type of tea and the subtypes of WTEA are overlooked. Indeed, literature almost completely ignores that not all WTEA is the same. Previous comparative studies from our group showed that commercial WTEA has a greater antioxidant potential and better health-promoting properties than commercial green tea. In this thesis we proposed to study the metabolic and oxidative effects of WTEA extracts subtypes BMD and BYHZ. These subtypes of WTEA are suggested to have similar phytochemical composition though their effects in health may be different as small changes in chemical composition may promote different effects in health. Our study supports previous findings in rat SCs, showing that WTEA extracts show great potential to improve the nutritional support of spermatogenesis without compromising the redox homeostasis. In our preliminary study to identify a concentration suitable for the subsequent studies, we identified WTEA extract subtype BMD as cytotoxic for hSC in a concentration of 5 mg/mL. This is a very high concentration, but it is interesting to note that WTEA extract subtype BHYZ did not presented any cytotoxic effect even at that high concentration thus suggesting that it may be safer to use in a human context of food supplementation than WTEA extract subtype BMD. Further studies will be needed to test this hypothesis.

The nutritional support of spermatogenesis is pivotal for spermatogenesis. On one hand specific metabolites such as lactate and acetate are needed to developing germ cells originate spermatozoa; on the other hand the redox balance has to be well controlled to avoid the overproduction of ROS that can readily attack the membranes. Our data shows that both WTEA extract subtypes BMD and BHYZ increase glycolysis-related membrane transporters in hSC, while also decreasing LDH activity, probably to sustain the characteristic high glycolytic flux that these cells are known for. In addition, hSC exposure to both WTEA extract subtypes decreases mitochondrial complex V expression, which further indicates an adaptative mechanism to the metabolic changes detected. Indeed, protein carbonylation was also decreased after exposure to WTEA extract of both subtypes. Overall, these results illustrate that WTEA extract modulates metabolic and oxidative profile of hSCs, which appears to have a positive effect to the nutritional support of spermatogenesis.

The WTEA extract subtypes BMD and BHYZ have a distinct preparation and thus, phytochemical composition may be also different. Our aim was also to compare the effect of both extracts, attained directly from China with a collaboration with Hangzhou University, in hSCs. Interestingly, WTEA extract subtype BMD presented a cytotoxic effect for hSC when in high concentrations but had relevant effects in the concentration of 0.5 mg/mL. At that concentration, this subtype of WTEA decreased oxidative stress (as detected by studying the lactate/alanine ratio) which was followed by decreased mitochondrial DNA copy number and increased mitochondrial complex II activity. Overall, our data suggests that this subtype of

WTEA may be more effective to counteract deleterious effects induced by metabolic diseases that alter the nutritional support of spermatogenesis. Nevertheless, more studies will be needed to support this hypothesis. Better understanding of the mechanisms by which WTEA, and its subtypes, may improve the metabolic and oxidative profile of the somatic SCs will unveil the potential for these compounds to be used in the production of dietary supplements to avoid subfertility or even infertility, particularly when associated with metabolic diseases.

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