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

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ABSTRACT

Sertoli cells are crucial for the success of spermatogenesis, which is the biological process that ensures male fertility. These cells present high metabolic rates, being often subjected to high oxidative stress levels that, if uncontrolled, may compromise male fertility. Since the most abundant tea catechin, epigallocatechin-3-gallate (EGCG), has demonstrated a potent preventive activity against oxidative stress, we have evaluated its effect at concentrations of 5 and 50 μM, on the metabolism, mitochondrial functionality and oxidative profile of human Sertoli cells (hSCs). While, the highest concentration of EGCG (50 μM) increased glucose and pyruvate consumption, it decreased the conversion of pyruvate to alanine to sustain a regular lactate production. However, despite maintaining Krebs cycle functionality, EGCG (50 μM) decreased mitochondrial membrane potential of hSCs, which could compromise the normal rates of ATP production. Interestingly, oxidative damages to proteins and lipids decreased in this experimental group, which may be valuable for the nutritional support of spermatogenesis.

1. Introduction

Tea is one of the most widely consumed beverages worldwide. Although it is commonly drunk for pleasure, tea is well-known for its health-promoting properties. The main constituents of tea leaves include proteins, polyphenols, methylxanthines, amino and organic acids (Dias et al., 2014c). Epigallocatechin-3-gallate (EGCG) is the most abundant and biologically active tea polyphenol, representing 50–80% of the total tea catechins (Khan and Mukhtar, 2007). Most of the tea's medicinal properties are attributed to EGCG, which has demonstrated a potent preventive activity against oxidative stress (OS) (Lombardo et al., 2011). Its ability to protect cells from reactive oxygen species (ROS)-induced damages has made EGCG a popular nutraceutical. Tea and its constituents are currently receiving considerable attention since they can be used as dietary supplements to prevent or even treat several diseases/dysfunctions. For instance, they have been described as potential modulators of spermatogenesis (Dias et al., 2014a), which is the process of sperm production. Sertoli cells (SCs) are the testicular cells responsible for the maintenance of spermatogenesis. The resulting metabolites of the distinctive metabolism of these cells are essential for the survival of developing germ cells. Particularly, the lactate produced by SCs constitutes the preferred substrate of germ cells for energy production (Dias et al., 2014b). When the metabolic cooperation between SCs and germ cells is disrupted, fertility problems may arise (Alves et al., 2014). The increasing incidence of male subfertility/infertility highlighted the need for new therapies. Interestingly, the consumption of tea seems to be a promising approach (Dias et al., 2016b; Oliveira et al., 2015b). However, the underlying mechanisms remain unknown. We have previously demonstrated that a white tea extract was able to alter rat SCs metabolism, suggesting possible implications for male fertility (Martins et al., 2014). Additionally, the exposure of human SCs (hSCs) to the most abundant methylxanthine of tea, caffeine, led to increased production of lactate and alanine, affecting

Abbreviations: EGCG, epigallocatechin-3-gallate; ETC, electron transport chain; GLUT1, glucose transporter 1; GLUT2, glucose transporter 2; GLUT3, glucose transporter 3; GLUTs, glucose transporters; hSCs, human Sertoli cells; LDH, lactate dehydrogenase; MCT4, monocarboxylate transporter 4; OS, oxidative stress; OXPHOS, oxidative phosphorylation; PFK1, phosphofructokinase 1; ROS, reactive oxygen species; SCs, Sertoli cells; SRB, sulforhodamine B.

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cells oxidative profile (Dias et al., 2015b). Tough, there is a lack of information concerning the effect of catechins on these testicular cells. Herein, we aimed to evaluate the effect of EGCG (5 and 50 μM) on hSCs metabolism, mitochondrial functionality and oxidative profile. We hypothesize that EGCG can act as a relevant modulator of the nutritional support of spermatogenesis, being useful to counteract male reproductive problems associated with hSCs dysfunction.

2. Material and methods

2.1. Chemicals

Dulbecco's Modified Eagle Medium (DMEM), Ham's F-12 (F12) and Fetal Bovine Serum (FBS): Biochrom (Leonorenstr, Berlin, Germany); M-PER Mammalian Protein Extraction Reagent and LDH Enzymatic Assay Kit: Thermo Scientific (Waltham, MA, USA); JC1: Molecular Probes (Eugene, OR, USA); EGCG (CAS Number 989-51-5): Sigma-Aldrich (St. Louis, MO, USA); Extracellular O_2 Consumption assay: Abcam (Cambridge, UK); All other chemicals: Sigma-Aldrich (St. Louis, MO, USA) unless specifically stated.

2.2. Human Sertoli cell primary culture

Testicular tissue processing was performed at Centre for Reproductive Genetics Professor Alberto Barros (Porto, Portugal) according to local, national, and European ethical committees' guidelines and the Declaration of Helsinki. Six testicular biopsies were obtained from six different patients with conserved spermatogenesis, after informed written consent. hSCs were obtained from the cells left in tissue culture plates after each patient's treatment and isolated by our routine method (Oliveira et al., 2009). Cells from each individual were plated separately in six cell + culture flasks (Sarstedt, Nümbrecht, Germany) and incubated at 33–34 °C to mimic the temperature to which hSCs are subjected *in vivo* into the scrotum (Ivell, 2007). Moreover, a controlled atmosphere of 5% CO_2 was maintained in the incubator, since it is required to stabilize the pH of the growth media. After 96 h, cultures were examined by phase contrast microscopy and only hSCs with contaminants below 5% were used. hSCs culture purity was determined as previously described (Alves et al., 2012).

2.3. Experimental groups

hSCs were allowed growing until reaching 90–95% of confluence. Subsequently, the culture media were replaced by serum-free media (DMEM:F12) supplemented with insulin–transferrin–sodium selenite (ITS) media (10 μg/mL–5.5 μg/mL–0.005 μg/mL, respectively). Three experimental groups were defined: a control group without EGCG and two groups supplemented either with 5 or 50 μM of EGCG. Although the bioavailability of EGCG after drinking tea is very low (0.3–0.5 μM) (Yang et al., 2009), the ingestion of 400–1200 mg of EGCG from a tea extract (fasting conditions) may result in plasma levels of 2–7 μM (Chow et al., 2003). Based on these studies, we have selected the lowest concentration of 5 μM of EGCG. Furthermore, since many studies reported the pharmacological relevance of EGCG at 50 μM (Albrecht et al., 2008; Weber et al., 2004), we have also included this concentration in our study. After 24 h of treatment with 0, 5 or 50 μM of EGCG, cells and the respective culture media were collected. Cell viability was evaluated by the Trypan Blue Exclusion test and averaged 85–90%.

2.4. Sulforhodamine B assay

A sulforhodamine B (SRB) colorimetric assay was performed as previously described (Fricker, 1994), to evaluate hSCs proliferative responses to the culture media (Dias et al., 2015a). In brief, hSCs were plated in a 96-well culture plate (same amount of cells per well), left to grow until reaching a confluence of 60–70% and treated with the test culture media containing 0, 5 or 50 μM of EGCG for 24 h. Then, cells were fixed overnight (at −20 °C) with a mixture containing 1% acetic acid and 99% methanol and subsequently stained with 0.05% (w/v) SRB dissolved in 1% acetic acid for 1 h. Unbound SRB was removed by washing with 1% acetic acid, whereas the bound SRB was solubilized with 10 mM Tris base (pH 10) in a shaker for 10 min. A blank was made with Tris base (pH 10) and the absorbance was read at 492 nm. Absorbance readings of SRB-stained cells gives a direct measure of cell numbers. To obtain concentration–response curves we defined the cell growth of the control group as 100% and calculated the cell growth of treated groups relative to control.

2.5. Proton NMR (^{1}H-NMR) spectroscopy

^{1}H-NMR spectra of hSCs extracellular culture media were acquired and quantified as described by our team (Alves et al., 2011). Sodium fumarate (final concentration of 1 mM) was used as an internal reference (6.50 ppm) to quantify the following metabolites present in hSCs extracellular media (multiplet, ppm): lactate (doublet, 1.33); alanine (doublet, 1.45), acetate (singlet, 1.90), pyruvate (singlet, 2.35), citrate (multiplet, 2.57–2.72) and H1-α-glucose (doublet, 5.22). Relative areas of ^{1}H-NMR resonances and metabolites concentrations were quantified as described (Alves et al., 2011).

2.6. Western blot

Total proteins from hSCs were isolated using M-PER Mammalian Protein Extraction Reagent according to manufacturer's instructions. Western blot was performed as previously described (Dias et al., 2013). In brief, proteins were fractionated in 12% polyacrylamide gels, then the separated proteins were transferred to previously activated polyvinylidene difluoride (PVDF) membranes and blocked for 90 min with a 5% non-fat milk solution at room temperature. Afterwards, the membranes were incubated overnight at 4 °C with the primary antibodies listed in Table 1. Mouse anti-β-actin was used as the protein loading control. The immunoreactive proteins were detected separately and visualized after incubation with the respective secondary antibodies (Table 1) for 90 min, at room temperature. The band density attained was divided by the corresponding β-actin band intensities and expressed in fold variation (induction/reduction) relative to the control group.

2.7. Lactate dehydrogenase (LDH) enzymatic assay

Intracellular LDH activity levels of hSCs were spectrophotometrically determined using the LDH Enzymatic Assay Kit as described (Dias et al., 2015b). Absorbance at 490 nm was measured using an Anthos 2010 microplate reader (Biochrom, Berlin, Germany). LDH enzymatic activities were calculated as units per milligram of protein, using the molar extinction factor (ε) and expressed as fold variation to the control group.

2.8. Mitochondrial membrane potential

The fluorescent probe JC1 was used to measure mitochondrial membrane potential of hSCs, using a slightly modified version of the method described by Salvioli et al. (1997). The accumulation of the JC1 dye in mitochondria depends on mitochondrial membrane potential. In brief, hSCs were cultured in a 96-well culture plate (same amount of cells per well) with DMEM:F12 (1:1, pH 7.4) supplemented with 1% FBS. Cells were left to grow until reach 60–70% of confluence. Then, the culture medium was replaced by ITS medium supplemented with 0, 5 or 50 μM of EGCG. After 24 h, the medium was removed and cells were washed with PBS. 100 μL of JC1 staining solution (1 μg/mL), previously prepared in DMEM:F12 supplemented with 1% FBS, were added to each well and cells were incubated for 15 min at 37 °C. Afterwards, cells were washed with PBS and 100 μL of DMEM:F12 supplemented

Table 1
List of the primary and secondary antibodies used in this study.

Antibody	Source	kDa	Dilution	Vendor	Catalog #
GLUT1	Rabbit	55	1:200	Santa Cruz Biotechnology Heidelberg, Germany	sc-7903
GLUT2	Rabbit	60–62	1:5000	Santa Cruz Biotechnology Heidelberg, Germany	sc-9117
GLUT3	Goat	48–70	1:200	Santa Cruz Biotechnology Heidelberg, Germany	sc-7582
PFK1	Rabbit	85	1:500	Santa Cruz Biotechnology Heidelberg, Germany	sc-67028
MCT4	Rabbit	43	1:1000	Santa Cruz Biotechnology Heidelberg, Germany	sc-50329
LDH	Rabbit	37–38	1:10,000	Abcam Cambridge, MA, USA	ab52488
β-Actin	Mouse	42	1:5000	Thermo Scientific Waltham, MA, USA	MA5-15739
DNP	Rabbit	–	1:5000	Sigma-Aldrich St. Louis, MO, USA	D9656
Nitro-tyrosine	Rabbit	–	1:5000	Cell Signaling Technology Leiden, Netherlands	9691
4-HNE	Goat	–	1:5000	Merck Millipore Temecula, USA	AB5605
Total OXPHOS cocktail	Mouse	CI-20 CII-30 CIII-48 CIV-40 CV-55	1:1000	MitoSciences Eugene, Oregon, USA	ab110413
Mouse	Goat	–	1:5000	Sigma-Aldrich St. Louis, MO, USA	A3562
Rabbit	Goat	–	1:5000	Sigma-Aldrich St. Louis, MO, USA	A3687
Goat	Rabbit	–	1:5000	Sigma-Aldrich St. Louis, MO, USA	A4187

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

with 1% FBS were added to each well. Fluorescence intensities were analyzed immediately using a Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek, Winooski, USA) pre-heated at 37 °C. Cells with functional mitochondria exhibited JC1 aggregates that were detected at 550/590 nm (excitation/emission), while cells with dysfunctional mitochondria mainly exhibited JC1 monomers detected at 485/535 nm (excitation/emission). The JC1 ratio aggregates/monomers was calculated for each condition as a measure of mitochondrial functionality.

2.9. Extracellular oxygen consumption assay

Oxygen consumption is one of the most informative and direct measures of mitochondrial function. We used the extracellular oxygen consumption assay kit (ab197243; Abcam, Cambridge, UK), according to the manufacturer's instructions, to measure the respiration of hSCs after exposure to EGCG. Briefly, hSCs were cultured in a 96-well culture plate with DMEM:F12 (1:1, pH 7.4) supplemented with 10% FBS. Cells were left to grow until reach 80–90% of confluence. Then, the medium was replaced by ITS medium supplemented with 0, 5 or 50 μM of EGCG. After 24 h of exposure, the medium was removed and replaced by freshly prepared ITS medium. 10 μL of Extracellular O_2 consumption reagent were added to each well, except to the blank control. 2 drops of high-sensitivity mineral oil (pre-heated at 37 °C) were added to each well to limit back diffusion of ambient oxygen. Fluorescence intensities were analyzed immediately using a Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek, Winooski, USA) pre-heated at 37 °C. Extracellular oxygen consumption of hSCs was measured at 1.5 min intervals for 120 min at excitation/emission = 380/650 nm. Cells respiration leads to oxygen depletion from the surrounding environment, resulting in the increase in fluorescence signal. Fluorescence intensities were normalized to the blank and expressed as counts per second (CPS) *versus* time (min).

2.10. Analysis of carbonyl groups, nitration and lipid peroxidation

Carbonyl groups, nitration and lipid peroxidation are usually used as biomarkers for oxidation and can be evaluated by measuring its resulting products, 2,4-dinitrophenyl (DNP), nitro-tyrosine and 4-hydroxynonenal (4-HNE), respectively. The content of these adducts in hSCs after exposure to EGCG was evaluated using specific antibodies (Table 1) by slot-blot as previously described (Dias et al., 2015b; Oliveira et al., 2015b). Results were expressed as fold variation to the control group.

2.11. Statistical analysis

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

3. Results

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

We have evaluated hSCs proliferation after exposure to the several doses of EGCG by the SRB assay. Our results demonstrated that addition of 5 μM of EGCG to hSCs culture medium slightly decreased the cell growth to 77 ± 15% comparative to control group (100 ± 16%) (Fig. 1). Additionally, the group of hSCs exposed to 50 μM of EGCG presented a cell proliferation of 34 ± 11%, illustrating a significant decrease to almost one-third of that observed in the control group (Fig. 1). Based on these results, all the subsequent results were normalized for the total number of cells or amount of protein in each sample.

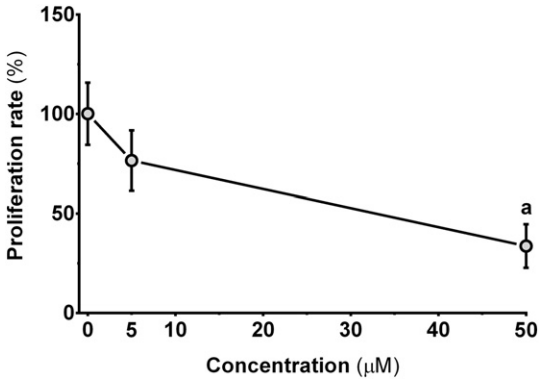

Fig. 1. Effect of epigallocatechin-3-gallate (5 and 50 μM) in the proliferation of human Sertoli cells (hSCs). The figure shows pooled data of independent experiments. hSCs growth is presented as percentage and control value was set at 100%. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control.

Table 2
Metabolites consumption/production and lactate/alanine ratio in human Sertoli cells from the control group and groups exposed to 5 or 50 μM of epigallocatechin-3-gallate (EGCG).

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

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

3.2. Glucose consumption by hSCs was stimulated by exposure to 50 μM of EGCG, though GLUT2 protein levels decreased

We have firstly evaluated glucose uptake since it is the main metabolic substrate of cultured hSCs. Our results showed a glucose consumption of 49 ± 16 and 47 ± 13 pmol/μg protein in non-exposed hSCs and those exposed to 5 μM of EGCG, respectively (Table 2). Contrastingly, there was an increase in glucose consumption to 161 ± 47 pmol/μg protein in hSCs exposed to 50 μM of EGCG, relative to cells from the other experimental groups (Table 2). Glucose transport through the hSCs cytoplasmic membrane is mediated by glucose transporters (GLUTs), particularly GLUT1, GLUT2 and GLUT3 (Oliveira et al., 2012). In our experimental conditions, we observed that neither the protein levels of GLUT1 nor GLUT3 (Fig. 2A) were altered in hSCs after exposure to EGCG. However, there was a decrease in GLUT2 protein levels in hSCs exposed to 50 μM of EGCG (0.56 ± 0.13 fold variation to the control) relative to non-exposed hSCs and those exposed to 5 μM of EGCG (1.15 ± 0.13 fold variation to the control) (Fig. 2A).

3.3. Exposure of hSCs to 50 μM of EGCG stimulated pyruvate consumption

Once glucose enters the cell, a cascade of reactions involving the enzyme phosphofructokinase 1 (PFK1), leads to the conversion of glucose to pyruvate (Martins et al., 2013). For that reason, we evaluated PFK1 protein levels, as well as pyruvate consumption by hSCs exposed to EGCG. Concerning PFK1 protein levels, no differences were found between the experimental groups (Fig. 3A). However, pyruvate consumption increased from 3.28 ± 0.36 pmol/μg protein in the control group to 6.25 ± 0.82 pmol/μg protein by hSCs exposed to 50 μM of EGCG (Table 2). Exposure of hSCs to 5 μM of EGCG led to a pyruvate consumption of 4.09 ± 0.40 pmol/μg protein, which was not significantly different relative to control (Table 2).

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

In hSCs, most of the pyruvate produced is converted to lactate by LDH (Dias et al., 2014b) and then exported to the intratubular fluid by the monocarboxylate transporter 4 (MCT4) (Galardo et al., 2007). Our results demonstrated a decrease in LDH protein levels in hSCs exposed to 50 μM of EGCG (0.56 ± 0.03 fold variation to the control) relative to hSCs from the control group and exposed to 5 μM of EGCG (1.08 ± 0.13 fold variation to the control) (Fig. 3A). In addition, LDH activity of hSCs exposed to 5 μM of EGCG was 0.91 ± 0.11 fold variation to the control, a similar value to cells from the control group (Fig. 3A). There was a decrease in LDH activity of hSCs after exposure to 50 μM of EGCG (0.06 ± 0.02 fold variation to the control) in comparison to the other experimental groups (Fig. 3A). Moreover, exposure of hSCs to this dose also led to decreased protein levels of MCT4 (0.67 ± 0.05 fold variation to the control) comparative to cells from the control group and those exposed to 5 μM of EGCG (1.05 ± 0.07 fold variation to the control) (Fig. 3A). Despite, hSCs lactate production was not affected by exposure to EGCG (Table 2).

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

Besides being converted to lactate, pyruvate can also be converted to alanine (Kaiser et al., 2005) or it can enter the mitochondria to originate acetyl-CoA (Dias et al., 2014b). Subsequently, acetyl-CoA can lead to citrate (Costello and Franklin, 2006) and/or acetate production (Yamashita et al., 2006). Our results showed that EGCG did not alter acetate nor citrate production by hSCs (Table 2). However, alanine production in hSCs exposed to 50 μM of EGCG was decreased (1.79 ± 0.50 pmol/μg protein), relative to the control group (3.13 ± 0.29 pmol/μg protein) and hSCs exposed to 5 μM of EGCG (3.74 ± 0.65 pmol/μg protein) (Table 2). We also evaluated the ratio lactate/alanine, which is an index of cellular redox state (Oliveira et al., 2012). hSCs exposed to 50 μM of EGCG presented an increased lactate/alanine ratio (23.3 ± 0.8) when compared to the control group (10.4 ± 0.6) and hSCs exposed to 5 μM of EGCG (11.0 ± 0.4).

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

Mitochondrial electron transport chain (ETC) is one of the main cellular generators of ROS. ETC includes four multi-subunit complexes (complexes I–IV), responsible for oxidative phosphorylation (Turrens, 2003). The electron transport generates an electrochemical proton gradient across the inner mitochondrial membrane, measured as mitochondrial membrane potential, which drives ATP synthesis by

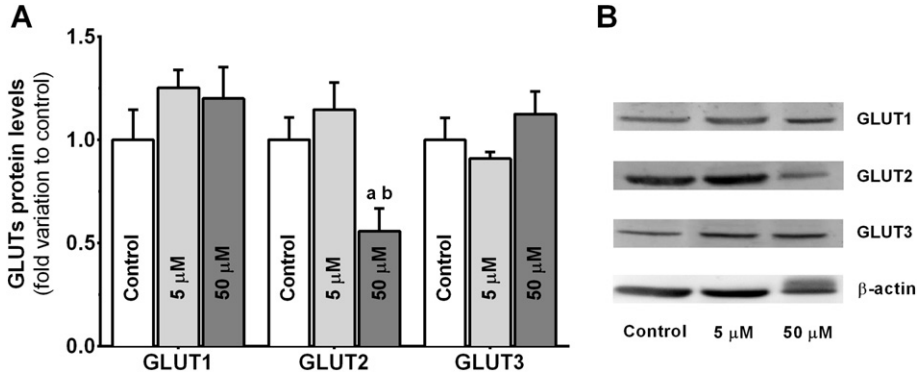

Fig. 2. Effect of epigallocatechin-3-gallate (5 and 50 μM) in glucose metabolism of human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating glucose transporters (GLUT1, GLUT2 and GLUT3) protein levels (Panel A). Panel B displays the representative blots (of one sample) of GLUT1, GLUT2 and GLUT3. Variation in protein levels is presented as fold variation to the control. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control; b – relative to 5 μM EGCG.

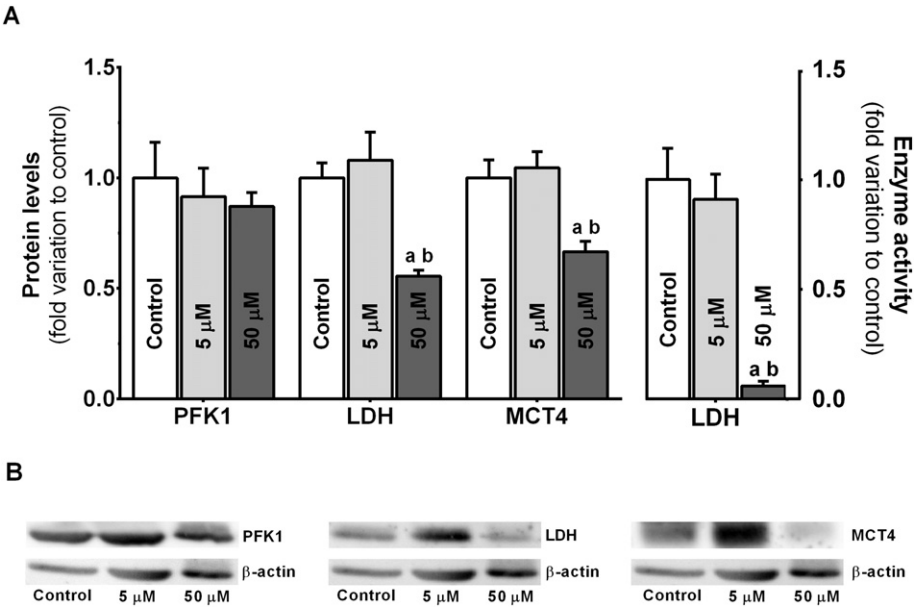

Fig. 3. Effect of epigallocatechin-3-gallate (5 and 50 μM) in pyruvate and lactate metabolism of human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating phosphofructokinase 1 (PFK1) protein levels, lactate dehydrogenase (LDH) protein levels and activity, as well as monocarboxylate transporter 4 (MCT4) protein levels (Panel A). Panel B displays the representative blots (of one sample) of PFK1, LDH and MCT4. Variation in protein levels is presented as fold variation to the control. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control; b – relative to 5 μM EGCG.

complex V (Lu and Cao, 2008). Our results did not show significant alterations in the protein levels of mitochondrial complexes I–V in hSCs exposed to EGCG (Table 3). However, JC1 ratio decreased after hSCs exposure to the highest dose of EGCG (50 μM) (1.05 ± 0.07) relative to control group (1.85 ± 0.14) and hSCs exposed to 5 μM of EGCG (1.80 ± 0.27) (Fig. 4A), showing a decrease in mitochondrial membrane potential in cells exposed to the highest dose of EGCG. Nevertheless, oxygen consumption was similar among the hSCs from the several experimental groups (Fig. 4B).

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

As high metabolic rates and mitochondrial activity are usually associated with ROS overproduction (Aitken et al., 2010) and EGCG is known for its antioxidant properties (Lombardo et al., 2011), we evaluated its effects against oxidative damages in hSCs. We quantified the formation of end-products resultant from protein oxidation or nitration, as well as lipid peroxidation when cells were exposed to 5 or 50 μM of EGCG. Our data showed decreased levels of carbonyl groups and nitration in hSCs exposed to 50 μM of EGCG (0.86 ± 0.04 and 0.80 ± 0.01 fold variation to the control, respectively) relative to cells exposed to 5 μM of EGCG (1.12 ± 0.10 and 1.08 ± 0.10 fold variation to the control, respectively) (Fig. 5). Exposure of hSCs to 50 μM of EGCG also led to decreased levels of lipid peroxidation (0.85 ± 0.04 fold variation to the control) comparatively to hSCs from the control group and those exposed to 5 μM of EGCG (1.06 ± 0.02 fold variation to the control) (Fig. 5).

Table 3
Protein expression levels of mitochondrial complexes in human Sertoli cells from the control group and groups exposed to 5 or 50 μM of epigallocatechin-3-gallate (EGCG).

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

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

4. Discussion

Oxidative damage to cells and biomolecules has been associated with the pathology of several diseases/dysfunctions, including male fertility (Makker et al., 2009). SCs are essential for the successful progression of spermatogenesis since they provide all the metabolic needs to the developing germ cells (Martins et al., 2013). Modulation of SCs metabolism is essential for spermatogenesis (Rato et al., 2012). Thus, modulators of SCs metabolism can be valuable to counteract male subfertility/infertility in some conditions, and tea constituents have shown promising results (Dias et al., 2015b; Martins et al., 2014). Studies focused on the role of EGCG in male fertility are scarce and mostly focused on its effects on sperm quality (De Amicis et al., 2012; Dias et al., 2016a). Since male fertility capacity is highly dependent on hSCs metabolism, we evaluated the effects of EGCG on those mechanisms. Despite the intricate limitations of the *in vitro-in vivo* data extrapolations concerning metabolism, we consider that our *in vitro* model possesses the most important features of SCs *in vivo* and that the data obtained would be a further step in understanding the effects of EGCG on SCs physiology and function (Reis et al., 2015).

EGCG can be orally consumed in food supplements prepared from very concentrated tea extracts or in infusions (diluted). Although the bioavailability of EGCG after drinking tea is very low (0.3–0.5 μM) (Yang et al., 2009), the ingestion of 400–1200 mg of EGCG from a tea extract (fasting conditions) may result in plasma levels of 2–7 μM (Chow et al., 2003). Thus, we selected 5 μM as the lowest concentration of EGCG in the study. As there are several studies reporting a pharmacologically relevant action of 50 μM of EGCG (Albrecht et al., 2008; Weber et al., 2004), we also evaluated the effects of that concentration as well. The main objective of investigating the effects of this supraphysiologic dose of EGCG is its possible usage in a specific treatment for male reproductive dysfunctions. We choose to expose hSCs to EGCG for 24 h to mimic a chronic, prolonged and repetitive dose of EGCG in a short-time.

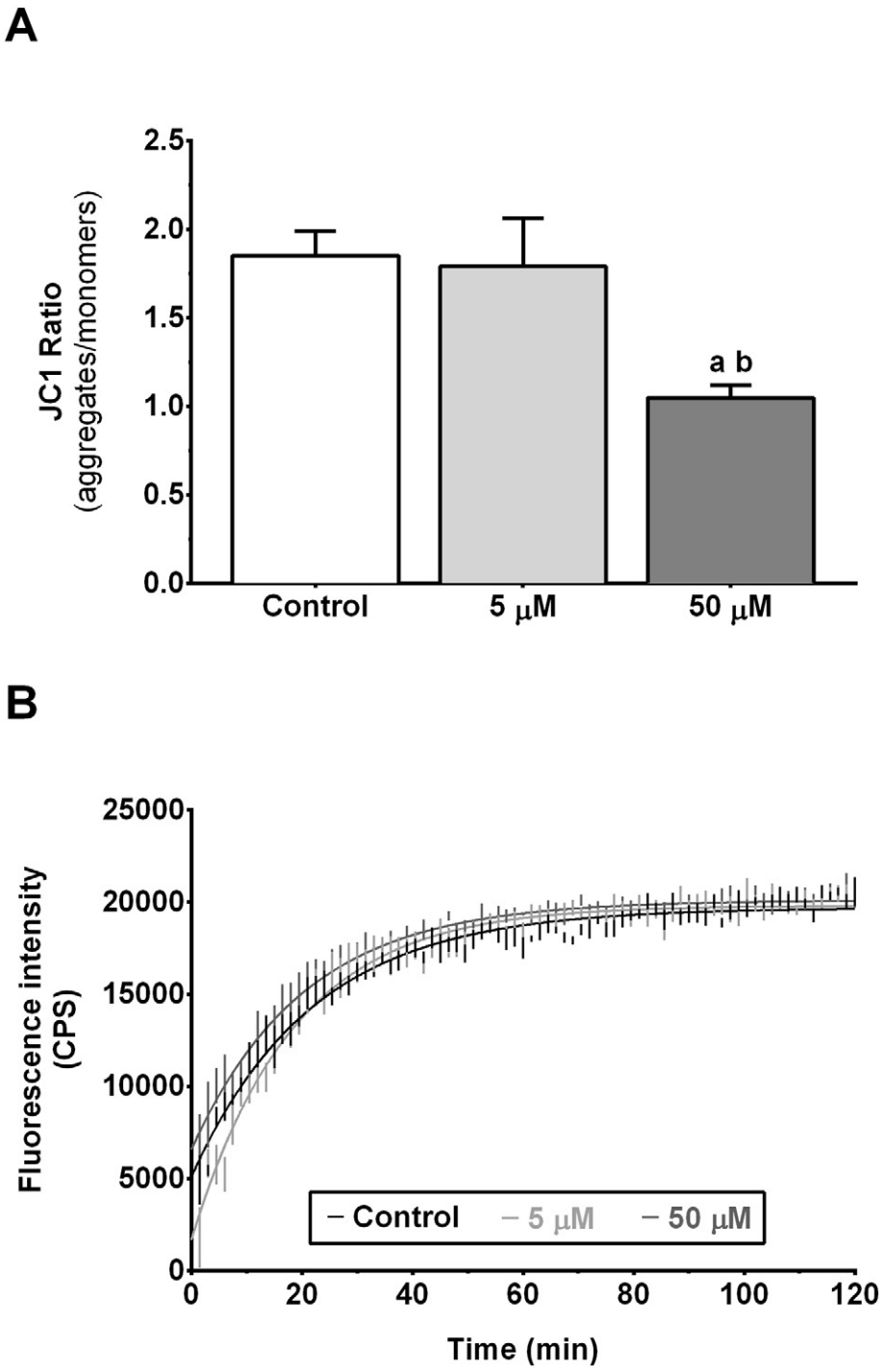

Fig. 4. Effect of epigallocatechin-3-gallate (5 and 50 μM) in mitochondrial membrane potential and extracellular oxygen consumption of human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating JC1 ratio (Panel A) and extracellular oxygen consumption (Panel B). Results of JC1 are presented as a ratio aggregates/monomers while oxygen consumption is presented as fluorescence intensity in CPS (counts per second). Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control; b – relative to 5 μM EGCG.

Most of EGCG studies report its cancer-preventive properties (Azam et al., 2004), particularly its ability to act as a potent inhibitor of cell proliferation (Kang, 2015). Previous studies have evidenced significant metabolic similarities between hSCs and cancer cells (for review (Oliveira et al., 2015a)). Still, studies from our research group have reported that the intensive glycolytic activity of hSCs is not always correlated with the proliferative properties of these cells (Dias et al., 2015a; Jesus et al., 2015; Martins et al., 2015), as frequently happens with cancer cells (for review (Oliveira et al., 2015a)). The results here presented demonstrated a dose-dependent decrease of hSCs proliferation when exposed to 5 and 50 μM of EGCG, evidencing a clear anti-proliferative effect of this compound on these testicular cells. This scenario might also be a result from EGCG cytotoxicity to hSCs. The exposure of hSCs to 50 μM of EGCG during 24 h might overcome the maximum tolerable dose, thus exerting toxicity to hSCs, which results in the observed decrease in hSCs proliferation. However, some caution should be taken when extrapolating these results to an *in vivo* situation due to the differences in the proliferating ability of Sertoli cells *in vitro* and *in vivo* (Reis et al., 2015).

EGCG has been ascribed as modulator of cells metabolism, including spermatozoa (De Amicis et al., 2012). To investigate the effects of EGCG

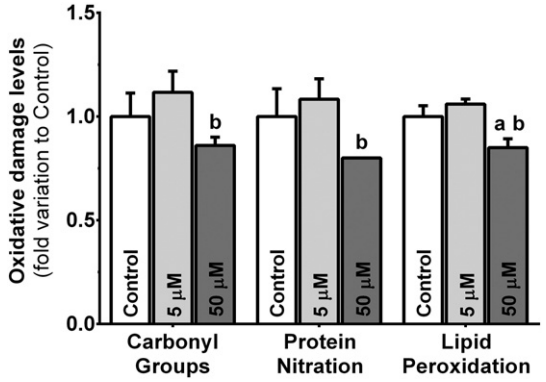

Fig. 5. Effect of epigallocatechin-3-gallate (5 and 50 μM) in oxidative damage levels of human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating carbonyl levels, protein nitration and lipid peroxidation. Results are presented as fold variation to control. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control; b – relative to 5 μM EGCG.

on the metabolic profile of hSCs, we selected key intervenient of the glycolytic pathway, since glucose is the main metabolic substrate of cultured hSCs and lactate is the key substrate produced by hSCs for developing germ cells (Rato et al., 2012). Exposure of hSCs to the highest concentration of EGCG (50 μM) increased glucose consumption by these cells. Notably, a similar effect induced by 40 μM of EGCG was reported in muscle cells (Zhang et al., 2010). In hSCs, glucose uptake is mediated by GLUT1, GLUT2 and GLUT3 (Oliveira et al., 2012). We have not detected any alteration in GLUT1 or GLUT3 protein levels. Though, there was a decrease in GLUT2 levels in hSCs exposed to 50 μM of EGCG. This suggests that the increased glucose uptake observed was sustained by GLUT1 and GLUT3, which are described to have a higher affinity for glucose than GLUT2, allowing cells to withstand a high rate of glucose transport (Wood and Trayhurn, 2003). Since GLUT2 presents a high Michaelis constant (K_m), its rate of glucose uptake decreases in parallel with the decrease of glucose concentration under the physiological range (Eny et al., 2008). Thus, the decrease in GLUT2 protein levels, can be a result of an adaptive response to the decrease in glucose concentration related to the consume of glucose present in the culture media (Macheda et al., 2005).

Once inside the cell, glucose is readily converted to pyruvate, in a process involving a rate-limiting step mediated by PFK1, which is a control point of the glycolytic pathway (Rato et al., 2013). The protein levels of this enzyme in hSCs were not altered by exposure to EGCG, suggesting that pyruvate production is not compromised. In hSCs, the great majority of pyruvate is transformed in lactate, by LDH (Dias et al., 2014b), and then transported to the extracellular compartment by MCT4, where it can be used by developing germ cells (Martins et al., 2013). Exposure of hSCs to 50 μM of EGCG led to a decrease in LDH protein levels and activity, and also to decreased MCT4 protein levels. Nevertheless, no differences were found in lactate production by hSCs exposed to this concentration of EGCG when compared with the other conditions. These results suggest that as lactate is being produced in normal amounts, LDH and MCT4 protein and/or enzyme activity levels are not rate limiting.

The maintenance of lactate production at control levels by hSCs exposed to 50 μM of EGCG is essentially due to the differences in pyruvate metabolism. Besides the higher pyruvate consumption, we also verified a decrease in alanine production in these cells. Since alanine is attained from pyruvate in a reversible reaction catalyzed by alanine transaminase (Miyashita et al., 2007) and its production is decreased in hSCs exposed to EGCG (50 μM), our results suggest that the higher pyruvate consumption is also responsible for the maintenance of the normal production of lactate. This scenario resulted in the increase of the lactate/alanine ratio in those cells. Lactate/alanine ratio is linked to the intracellular redox status, since it reflects the NAD^+/NADH ratio, which in turn is directly implicated in energy metabolism. It constitutes a metabolic node well suited for integration of energy metabolism and an optimal NAD^+/NADH ratio is essential for normal mitochondrial function (Alves et al., 2013).

The maintenance of pyruvate production by the glycolytic pathway under certain levels is also crucial to maintain a proper mitochondrial function, since it enters the mitochondria to be converted in acetyl-CoA (Kim, 1997). Then, acetyl-CoA can then enter the Krebs cycle, where it is converted to citrate (Costello and Franklin, 2006), or it can be exported to the cytosol and form acetate (Yamashita et al., 2006), which can be used for fatty acids and cholesterol synthesis (Shimazu et al., 2010). When there is a Krebs cycle truncation, citrate can also be transported to the cytosol or to extracellular compartment (Costello and Franklin, 2006). Since no alterations were found either in acetate or citrate extracellular production by hSCs after exposure to EGCG, we can suggest that pyruvate is not only being used to sustain lactate production, but also to fuel the Krebs cycle. These results support that the functionality of hSCs Krebs cycle is not being compromised by exposure to EGCG. The normal oxygen consumption among the experimental groups corroborates our data. In fact, if oxygen consumption was increased, oxidative metabolism should be occurring towards CO_2 production. Since oxygen consumption was not affected by exposure to EGCG and neither acetate nor citrate are being exported to the extracellular compartment, these two metabolites should be used for storage into lipid synthesis within the cytosol. This may be part of the additional nutritional support of spermatogenesis. However, we detected a decrease in mitochondrial membrane potential of hSCs exposed to 50 μM of EGCG. The same result was observed in a previous study where hepatocytes were exposed to 30 μM of EGCG (Kucera et al., 2015). Still, no differences were found in the protein levels of mitochondrial complexes I–V, which are responsible for oxidative phosphorylation (OXPHOS) and ATP synthesis (Turrens, 2003). Despite protein levels of mitochondrial complexes were not altered, EGCG may have some inhibitory effects on the activity of those complexes, as it was previously described (Valenti et al., 2013; Zheng and Ramirez, 2000). This may result in a mitochondrial uncoupling, as reported before (Lee and Kim, 2009), which may also be related to the cytotoxic effect of this dose. Although Krebs cycle homeostasis is not being compromised by exposure of hSCs to EGCG, we observed a dissipation of the mitochondrial potential, which might be associated with a reprogramming of the cellular metabolic pathways to sustain hSCs substrate requirements (Samudio et al., 2009). On the other hand, it is known that OXPHOS in mitochondria involves ROS production (Turrens, 2003) and it has been reported that EGCG can be accumulated in mitochondria modulating OS (Schroeder et al., 2009). In fact, there was a decrease in protein oxidation and nitration, as well as in lipid peroxidation in hSCs exposed to 50 μM of EGCG. This corroborates the protective role attributed to high doses of EGCG against ROS overproduction (Schroeder et al., 2009). Hence, EGCG seems to have a dual effect in hSCs mitochondrial function: while it decreases hSCs mitochondrial functioning, it also protects hSCs from ROS-induced damages due to its potent antioxidant potential.

5. Conclusions

Our study demonstrates a dose-dependent modulating action of EGCG in hSCs metabolism, mitochondrial functionality and oxidative profile. EGCG at high concentration (50 μM) could modulate hSCs metabolism, maintaining lactate production and Krebs cycle functionality. Despite 50 μM of EGCG might decrease ETC function, Krebs cycle process was preserved. Additionally, our data suggest that citrate and acetate are being used for storage into lipid synthesis within the cytosol, which might be part of an additional nutritional support for spermatogenesis. Moreover, 50 μM of EGCG was able to decrease oxidative damage to proteins and lipids, which may be of extreme importance in the improvement of spermatogenesis and male fertility, since oxidative stress is on the basis of several fertility problems (Agarwal et al.,

2008). The strong enhancement of glucose uptake verified in hSCs exposed to EGCG supports previously reported benefits of EGCG against Diabetes Mellitus (Chen et al., 2009; Ortsater et al., 2012), which is characterized by glucose intolerance (Rato et al., 2015) and strongly affects hSCs function (Dias et al., 2014b). Although the dose of 50 μM of EGCG might not be physiological attained by ingestion of EGCG-rich products, it could be attained with a local administration, supplements or certain medications. Thus, the main objective using this supraphysiologic dose was indeed to test the possible usage of EGCG in a specific treatment for male reproductive dysfunctions. Besides, the bioavailability of EGCG to Sertoli cells *in vivo* should be further investigated. Herein, EGCG is proposed as a novel modulator of cultured hSCs metabolic and oxidative profiles, which may have important effects in the nutritional support of spermatogenesis, particularly under unfavorable conditions, such as those evidenced by men with metabolic diseases.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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