TNF- α -induced oxidative stress and endothelial dysfunction in EA.hy926 endothelial cells is prevented by yerba mate and green coffee extracts and the microbial metabolite, dihydrocaffeic acid

Running title: Mate and coffee extracts, chlorogenic and dihydrocaffeic acid prevent TNF α effects in endothelial cells

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Abbreviation list: 5-caffeoylquinic acid (5-CQA); carbonyl groups (CG); chlorogenic acids (CGAs); cardiovascular disease (CVD); dihydrocaffeic acid (DHCA); dihydroferulic acid (DHFA); endothelial nitric oxide synthase (eNOS); glutathione peroxidase (GPx); glutathione reductase (GR); reduced glutathione (GSH); green coffee bean extract (GCBE); nitric oxide (NO); reactive oxygen species (ROS); tumor necrosis factor- α (TNF- α); yerba mate extract (YME).

Abstract

The main phenol in mate and coffee, 5-caffeoylquinic acid (5-CQA), and its major microbial metabolites, dihydrocaffeic (DHCA) and dihydroferulic (DHFA) acids, have shown oxidative-stress protective effects in HepG2 cells. To evaluate possible endothelial-protective effects of the extracts and compounds, endothelial EA.hy926 were pretreated with yerba mate (YME) and green coffee bean (GCBE) phenolic extracts, 5-CQA, DHCA and DHFA, and afterwards stressed with tumor-necrosis-factor-alpha (TNF- α) and then oxidative-stress markers and endothelial-nitric-oxide-synthase levels were studied. TNF- α (10ng/mL, 24h) depleted reduced glutathione (GSH) and eNOS levels, increased reactive oxygen species (ROS) production, glutathione peroxidase (GPx) and reductase (GR) activities, and protein oxidation (carbonyl groups, CG) in EA.hy926 cells. Pre-treatment with YME, GCBE, 5-CQA, DHCA at certain physiological concentrations, lowered ROS production, recovered depleted GSH, reduced GR and GPx activities, and CG levels, but enhanced eNOS. YME, GCBE and 5-CQA show antioxidant effects in endothelial cells playing DHCA an important role in such protection; moreover the extracts, 5-CQA, DHCA and DHFA increased eNOS levels.

Keywords: Yerba mate, green coffee, microbial metabolites, EA.hy926 cells, TNF- α

INTRODUCTION

Yerba mate has been extensively consumed by large populations in South America for centuries and is becoming popular worldwide because of its antioxidant, vaso-dilating, lipid-reducing, anti-glycation, anti-inflammatory, and weight-reducing properties, among other health beneficial effects (Bracesco et al, 2011). Many of these properties have been attributed to its rich content in phenolic compounds, which amount up to 8.1-9.8% in mate dry weight (Bravo et al, 2007). Mate is particularly rich in caffeoyl derivatives [caffeic acid, 3-, 4-, and 5-caffeoylquinic acids (5-CQA), 3,4-, 3,5-, and 4,5-dicaffeoylquinic acids], generically named chlorogenic acids (CGAs), accounting for over 90% of the total phenolic content, and in addition, it contains flavonoids (quercetin, rutin and kampherol glycosides; Bravo et al, 2007). Recently, a quantitative analysis carried out in different commercial mate brands describes that 3-caffeoylquinic (26.8–28.8%), 5-caffeoylquinic (21.1–22.4%), 4-caffeoylquinic (12.6–14.2%) and 3,5-dicaffeoylquinic acids (9.5–11.3%) along with rutin (7.1–7.8%) are the most abundant polyphenols (Mateos et al, 2018).

Coffee is more broadly consumed worldwide than yerba mate, in fact it is the most consumed beverage in the world. Green coffee beans, which are obtained from the cherries of the *Coffea* plant, are also rich in CGAs that may amount up to 4.1–11.3% (w/w; Perrone et al, 2010). The main phenolic compounds in green coffee beans are very similar to those in yerba mate: 3-, 4-, and 5-CQA, 3,4-, 3,5-, and 4,5-DCQA acids, and also 3-, 4-, and 5-feruloylquinic acids (Alonso-Salces et al, 2009; Baeza et al, 2016a, 2016b).

Cardiovascular diseases (CVD) are responsible for the highest rate of death around the world. One of the major complications in CVD is a chronic inflammation

process based on the stiffness of the arteries, atherosclerosis and endothelial dysfunction, which is considered an important early biomarker for the development of CVD. Oxidative stress has been suggested to play a critical role in the development of endothelial dysfunction, possibly via reduced bioavailability of nitric oxide (NO; Jiang et al, 2016) generated in endothelial cells from L-arginine by means of the endothelial nitric oxide synthase (eNOS). NO is the main factor responsible for vasodilation and maintenance of vascular tone, among other antiatherogenic effects (Andriantsitohaina et al, 2012) and thus, the importance of the endothelium as a central regulator of vascular homeostasis is increasingly recognized. Enhanced oxidative stress leads to imbalance of NO system, with decreased NO bioavailability and paradoxical oxidant generation by endothelial NOS (Grassi et al, 2015). In contrast, alleviation of oxidative stress has been shown to improve endothelial dysfunction (Jiang et al, 2016).

Polyphenols may reduce endothelial cell vulnerability to oxidative stress at both the membrane and cytosol level (Carluccio et al, 2003; Barringer et al, 2011) and stimulate eNOS through the activation of the estrogen receptor- α of endothelial cells via the inhibition of the p38 MAPK and PI3K/AKT pathways (Anter et al, 2005; Kane et al, 2009). CGA is a potent antioxidant *in vitro*, directly scavenging reactive oxygen species (Baeza et al, 2014), although this process is unlikely to occur *in vivo* being more probable that CGA and other dietary polyphenolic compounds act by boosting antioxidant enzymes for damage removal and repair systems (Forman et al, 2014). A recent study has shown that CGA is able to attenuate endothelial dysfunction, in part via oxidative stress/Hmox-1 pathway (Jiang et al, 2016). However these effects were observed using supraphysiological (i.e., 5 μ M, 10 μ M) doses of CGA (Jiang et al, 2016). In macrophages, phenols in mate may inhibit nuclear translocation of the transcription factor NF- κ B, suppressing the expression of pro-inflammatory cytokines

(Puangpraphant et al, 2011). In high-fat fed obese animals, mate has also shown to reduce metabolic and inflammatory markers (Arcari et al, 2011; Borges et al, 2013; Pimentel et al, 2013; da Silva et al, 2014) and in humans, to inhibit plasma and lipoprotein oxidation and reduce plasma levels of oxidative stress biomarkers (da Silva et al, 2008; Boaventura et al, 2012). Regarding the effects of acute or habitual consumption of coffee beverages on endothelial function, there is controversy. Focusing on single ingestion of coffee; several studies described improved endothelial function (Buscemi et al, 2009; Buscemi et al, 2010) whereas others reported no effect (Mubarak et al, 2012). The reasons for the discrepant responses seem to be the CGA dose in the beverages (Buscemi et al, 2010). Recently, the ingestion of coffee polyphenols showed to improve peripheral endothelial function after glucose loading in healthy subjects pointing to CGAs as the candidates responsible for this effect (Ochiai et al, 2014).

Most of aforementioned *in vitro* studies have been performed using coffee or mate phenolic extracts or 5-CQA at supraphysiological concentrations. However, CGAs are poorly bioavailable and less than 30% of the ingested dose is excreted in urine after extensive metabolism (Stalmach et al, 2009; Duarte & Farah, 2011; Renouf et al, 2014). Major CGAs metabolites are dihydrocaffeic (DHCA) and dihydroferulic (DHFA) acids, which are generated by the gut microbiota, reaching μM concentrations in plasma 5-10 h after the intake of CGAs (Stalmach et al, 2009; Renouf et al, 2014; Renouf et al, 2010; Gomez-Juaristi et al, 2018a; 2018b) and having been observed in urine 48 h after the intake of a single dose of coffee, thus with a long half-life (Duarte & Farah, 2011). Previous studies in our group have shown that DHCA, even at low physiologically relevant concentrations, strongly contributed to the antioxidant activity of yerba mate against a pro-oxidant agent in HepG2 cells (Baeza et al, 2016). To better understand the potential protective effects of yerba mate and coffee polyphenols on the vasculature, we

investigated the effects of major metabolites observed in plasma after the intake of these beverages, DHCA and DHFA, as well as 5-CQA, at a wide range of concentrations, including physiological. The effects have been studied in comparison to the yerba mate and green coffee extracts; although the extracts do not come into direct contact with the cells in vivo, we wanted to determine to what extent the metabolites retain the biological activity of the phenolic parent molecule and precursor extracts, and also because this would allow the comparison with other in vitro experiments carried out with the same extracts and compounds in other cell models.

MATERIALS AND METHODS

Reagents

Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were from Bio Whittaker Europe (Lonza, Madrid, Spain). 5-caffeoylquinic acid (5-CQA), gentamicin, penicillin, streptomycin, *o*-phthalaldehyde (OPT), glutathione reductase (GR), reduced (GSH) and oxidized (GSSG) glutathione, nicotine adenine dinucleotide (reduced) (NADH), nicotine adenine dinucleotide phosphate reduced salt (NADPH), 2',7'-dichlorofluorescein diacetate (DCFH-DA), 2,4-dinitrophenylhydrazine (DNPH), β -mercaptoethanol, guanidine, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), dimethylsulphoxide (DMSO) and sodium dodecyl sulphate (SDS) were purchased from Sigma-Aldrich (Madrid, Spain). Bradford reagent was acquired from Bio-Rad (Madrid, Spain). Recombinant murine TNF- α was from PreproTech (Tebu-bio, Madrid, Spain). eNOS ELISA kit was from R&D Systems (Materlab, Madrid, Spain). Cell culture dishes were from Falcon (Cajal, Madrid, Spain). Human umbilical vein EA.hy926 cells were kindly provided by Centro de Investigaciones Biológicas (CIB-CSIC, Madrid, Spain). All other chemicals were of analytical grade.

Preparation of yerba mate extract (YME), green coffee bean extract (GCBE) and pure compounds to test in cell culture

Yerba mate (*Ilex paraguariensis* St. Hil.) tea was obtained in a local supermarket in Madrid (Spain). It contained dry mate leaves together with some stems. Extracts from the mate tea were obtained from the commercial product without further milling or selection of the leaves. Green coffee (*Coffea arabica* L.), from Colombia, was purchased in a local supermarket in Madrid (Spain). Due to their hardness, the green coffee beans were milled first in a domestic coffee grinder and then in a mill to a final particle size of 0.5 μm .

The phenolic compounds were extracted from yerba mate and green coffee beans in triplicate by organic solvent extraction following the protocol described by Bravo and Saura Calixto (1998). Briefly, 1 g of sample was mixed with 40 mL of 2 N HCl in aqueous methanol (50/50, v/v) using an orbital shaker at room temperature for 60 min, and centrifuged (3000 g, room temperature, 10 min). The supernatant was collected and the pellet was re-extracted with 40 mL of 70% (v/v) acetone using an orbital shaking at room temperature for 60 min and centrifuged (3000 g, room temperature, 10 min). The supernatant was collected. Yerba mate (YME) and green coffee bean extracts (GCBE) were obtained by combining the corresponding supernatants from both extractions and making them up to 100 mL. The extracts were evaporated, lyophilized, and stored at -20°C for further use. Pure standards 5-CQA, DHCA, and DHFA were dissolved in 10% DMSO in deionized water to prepare 10 mM stock solutions. The characterization of the coffee and mate extracts here studied is described in Baeza et al (2016a, 2016b) and Mateos et al (2018), respectively. In a

previous study by our group, caffeine did not protect against oxidative stress in HepG2 cells (Baeza et al, 2014), therefore, the methylxanthine was not further studied in this work.

EA.hy926 cell culture and treatment

For this study, human vascular endothelium EA.hy926 cells were employed as a model of endothelial cells to study the protective effect of GCBE, YME, 5-CQA, DHCA, and DHFA against inflammation induced by TNF- α . EA.hy926 cells were grown in DMEM medium (4.5 g/L glucose) supplemented with 10% FBS and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin. EA.hy926 cells were maintained in 100 mm culture dishes in a humidified incubator containing 5% CO₂ and 95% air at 37°C. The culture medium was changed every other day in order to remove the non-adherent and dead cells, and the cells were usually split 1:3 when they reached confluence. To avoid potential differences in cellular shape and response due to confluency, all cells were usually treated at 60-70% confluency. The day before the experiments, cells were changed to 2% FBS medium to reduce potential FBS interference with the assays not altering cell growth (Hou et al, 2011).

For EA.hy926 cells treatments, YME and GCBE stock solutions of 100 mg/mL in 10% (v/v) DMSO were prepared, aliquoted and stored at -20°C. The YME and GCBE stock solution were diluted the day of the experiment with 2% FBS DMEM medium to prepare fresh working solutions at concentrations of 1, 20 and 50 μ g/mL in 0.1% DMSO. 5-CQA, DHCA and DHFA stock solutions (10 mM) were diluted with 2% FBS medium to prepare solutions at concentrations of 0.1, 1, 10, and 20 μ M 5-CQA in 0.1% DMSO, or 0.1, 0.3, 1, and 10 μ M DHCA and DHFA in 0.1% DMSO. The

extract and compound concentrations used in the assays were selected based on previous cell culture studies carried out in our research group (Baeza et al, 2014; 2016a; 2016b, 2017). In addition, peak plasma 5-CQA concentration of 1.5 μ M has been reported after the intake of a single serving of a coffee beverage with a medium (635 μ mol) chlorogenic acid content,⁷ whereas higher doses (1 – 40 μ M) were tested in cell cultures and found non-toxic (Baeza et al, 2014).

The potential effect on EA.hy926 cells of GCBE, YME and its main hydroxycinnamic acid 5-CQA and metabolites DHCA and DHFA was studied in direct and protective experiments. In the direct experiments, cells were incubated with YME, GCBE (1, 20 and 50 μ g/mL in 0.1% DMSO), 5-CQA (0.1, 1, 10, and 20 μ M in 0.1% DMSO), DHCA and DHFA (0.1, 0.3, 1, and 10 μ M in 0.1% DMSO) for 24 h (except for ROS experiments). In the latter, to study the protective effects of the tested compounds, EA.hy926 cells were stimulated with the pro-inflammatory cytokine TNF- α , so that after pre-treatment with the same concentrations of the YME, GCBE, 5-CQA, DHCA and DHFA for 20 h, the medium was discarded, cells were washed with PBS and incubated with 10 ng/mL TNF- α for 24 h.

Crystal violet assay

Cell viability was determined by the crystal violet assay (Kane et al, 2009). This method is based on the use of crystal violet, which is an acidophilic stain that accumulates in the cell nucleus and it correlates with the nuclear DNA content, and thus with viable cell number. Cells were seeded (10^4 cells/well) in 96-well plates; after 24 h incubation, cells were treated with the different compounds for 24 h, washed with 200

μL PBS and incubated with 50 μL of 0.2% (w/v) crystal violet solution (in 2%, v/v, ethanol) for 20 min. Wells were washed twice with 200 μL sterile Milli-Q water to remove excess of staining, and then 100 μL of 1% sodium dodecyl sulphate (SDS) were added to break down the cells and release the dye to estimate cell viability. The absorbance at 560 nm was measured using a microplate reader (Bio-Tek, Winooski, VT, USA). Results are expressed as percentage of cell viability referred to the absorbance measured in control cells.

Cell cytotoxicity

Cytotoxicity was determined by the lactate dehydrogenase (LDH) assay (Welder & Acosta, 1994). LDH is an intracellular enzyme and its release to the culture medium indicates cell damage. Cells (1.5×10^6 cells per plate) were seeded in 60 mm diameter plates and treated with 3 mL of the different concentrations of extracts and compounds as indicated in section 2.3. After treatment, the culture medium was collected; separately, the cells were scraped in 3 mL of PBS, sonicated to ensure the breakdown of the cell membrane and the release of the total amount of LDH, and centrifuged at 420 g at 4°C for 10 min. To determine LDH levels, 200 μL of 1.35 M Tris, 0.08 M pyruvate and 2 mg/mL NADH were added to 40 μL of culture medium or supernatant cell content in a 96 well plate and the absorbance at 340 nm was immediately read in a microplate reader for 5 min. The percentage of LDH leakage was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content.

Determination of ROS production

Cellular ROS were quantified by the dichlorofluorescein (DCF) assay using a microplate reader to screen the antioxidant effect of the different concentrations of each of the compounds studied (Alia et al, 2006). This assay is based on the measurement of the fluorescence produced by oxidation of the fluorescent probe DCFH-DA due to reactive oxygen species. Briefly, cells were seeded in 24-well plates (2×10^5 cells per well) in medium containing FBS and replaced with the FBS-free medium the following day. To evaluate the direct effect of the studied compounds, 20 h after seeding the cells, 10 μ L of DCFH-DA (10 μ M in DMSO) were added to the wells and cells were incubated at 37°C for 30 min thus DCFH-DA could penetrate into the cells. Then, cells were washed with PBS (0.5 mL) and placed in fresh FBS-free medium with the different concentrations of extracts and compounds. ROS production was monitored for 24 h by measuring the fluorescence in a microplate reader at 485 and 530 nm excitation and emission wavelengths, respectively. To evaluate the protective effect of the studied compounds, cells were seeded (2×10^5 cells per well) and left overnight before treating them with the different extracts and compounds in FBS-free medium for further 20 h. Then, 10 μ L of DCFH-DA (10 μ M in DMSO) were added to the wells and cells were incubated at 37°C for 30 min. Cells were washed with PBS (0.5 mL) and FBS-free medium with TNF- α was added to every well but controls with further incubation for 1 h. Control cells without TNF- α treatment were used as a negative control. Control cells not treated with extracts or pure compounds but incubated with TNF- α were used as a positive control. Fluorescence was read in a microplate reader at 485 and 528 nm excitation and emission wavelengths, respectively. Results are expressed as percent of fluorescence units from negative control.

Determination of GSH content

GSH, a tripeptide and one of the major endogenous antioxidant defenses of cells, was quantified by the fluorimetric assay developed by Hissin & Hilf (1976) with modifications. The method takes advantage of the reaction of reduced GSH with OPT at pH 8.0 to produce a highly fluorescent compound, which is measured at 340 and 460 nm excitation and emission wavelengths, respectively. Cells were plated in 60 mm diameter plates at a concentration of 1.5×10^6 cells/plate. Cells were treated with the different concentrations of extracts and compounds for 20 h, collected by scraping in 1.5 mL of PBS and centrifuged (420 g, 4°C, 5 min). Cellular lysates were obtained by adding 110 μ L of 5% (w/v) trichloroacetic acid containing 2 mM EDTA. Following centrifugation of cells at 5500 g and 4°C for 30 min, 50 μ L of the clear supernatant were transferred to wells in a 96-well plate. Then, 15 μ L of 1 M NaOH were added to neutralize the sample and 175 μ L of 0.1 M sodium phosphate buffer, pH 8.0, containing 5 mM EDTA and finally 10 μ L per well of a stock solution of OPT (10 mg/mL) in methanol were added. After 15 min at room temperature in the dark, fluorescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm. The results of the treatments were referred to those of a standard calibration curve of GSH.

Determination of glutathione peroxidase and glutathione reductase activities

GPx and GR are involved in the GSH turnover as enzymatic antioxidant cell defenses. GPx and GR activities were determined following the protocols described by Alía et al (2005). Briefly, cells were seeded in 100 mm diameter plates (2×10^6 cells/plate) and treated with the different extracts and standards for 20 h. Then cells

were scraped and collected in 1.5 mL of PBS and centrifuged at 420 g, 4°C for 10 min. Cell pellets were resuspended and sonicated in 200 µL of 50 mM Tris containing 5 mM EDTA and 0.5 mM β -mercaptoethanol. Cell lysates were obtained by centrifuging at 1200 g, 4°C for 15 min.

Determination of GPx activity was based on the oxidation of GSH by GPx, using *Tert*-butylhydroperoxide as a substrate, coupled to the disappearance rate of NADPH by GR. GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione (GSSG). Results are expressed as mU/mg protein. Protein concentration in the samples was measured using the Bio-Rad Protein assay reagent following manual's instructions.

Determination of protein damage

Carbonyl groups were determined as a biomarker of protein damage following the protocol described by Martín et al (2013). Cells were seeded in 100 mm diameter plates (2×10^6 cells/plate) and subjected to the different treatments. Afterwards, cells were scraped and collected in PBS. Cell pellets were obtained by centrifuging at 420 g, 4°C, 10 min, resuspended and sonicated in 110 µL of 0.25 M Tris buffer pH 7.4 containing 0.2 M sucrose and 5 mM dithiotreitol (DTT). The cytoplasmic content obtained by centrifugation was incubated with 4 volumes of 10 mM DNPH, and another aliquot treated with the same volume was incubated in 2 M HCl as control. All the tubes were kept in the darkness for 1 h at room temperature, mixing several times during incubation. The protein content was precipitated with the same volume of 10% trichloroacetic acid. After centrifuging and washing twice with 1 mL ethyl acetate: ethanol (1:1, v/v), the pellet was resuspended in 500 µL of 6 M guanidine and the

absorbance was measured at 360 nm. Results are expressed as nmol carbonyl/mg protein. Protein concentration was determined as aforementioned.

Analysis of human endothelial nitric oxide synthase levels

EA.hy926 cells were plated in 60 mm diameter plates at a density of 1.5×10^6 cells/plate. To evaluate the direct effects, cells were incubated with YME, GCBE, 5-CQA, DHCA and DHFA for 24 h at the aforementioned concentrations. To study the protective effects against TNF- α , cells were pre-treated with the same concentrations of YME, GCBE, 5-CQA, DHCA, and DHFA for 20 h, then the medium was discarded, cells were washed with PBS and stimulated with 10 ng/mL TNF- α for 24 h. Cells were collected by scraping in 1.5 mL of PBS and centrifuged (420 g, 4°C, 5 min). Then, cells were resuspended in PBS, sonicated and centrifuged (15300 g, 4°C, 30 min). The supernatants were collected, and protein concentration was determined using the Bio-Rad protein assay reagent. Cell lysates were aliquoted and stored at -80 °C until eNOS measurement.

Concentration of eNOS in cell lysates was determined using a human eNOS ELISA kit (R&D Systems) following the manufacturer's instructions. A calibration curve, in a range of concentration from 78.1 to 5000 pg/mL, was also run in each plate using recombinant human eNOS standard. Results were expressed as pg eNOS/mg protein.

Statistics

SPSS version 23.0 program (SPSS Inc., IL, USA) was used for the statistical analysis of data. Homogeneity of variances was checked by Levene test. As variances were homogenous, multiple comparisons were carried out using one-way ANOVA followed by Bonferroni test. The level of significance was set at $p < 0.05$. Results are expressed as mean $\pm$ standard deviation (SD).

RESULTS

Direct effects of YME, GCBE, 5-CQA, DHCA, and DHFA in viability, cytotoxicity and antioxidant effects in EA.hy926 cells

Table 1 shows the viability and cytotoxicity results obtained in basal conditions after direct treatment of EA.hy926 cells with YME, GCBE (1, 20 and 50 $\mu\text{g/mL}$), 5-CQA (0.1, 1, 10, and 20 μM), DHCA and DHFA (0.1, 0.3, 1, and 10 μM) for 24 h using the crystal violet and LDH assays, respectively. No statistically significant changes were found in cell viability, indicating that the concentrations selected for the study did not damage cell integrity during the period of incubation, neither cytotoxic effects were observed. Moreover, Table 2 shows that EA.hy926 cells exposed to YME at 20 $\mu\text{g/mL}$, GCBE at 20 and 50 $\mu\text{g/mL}$, 5-CQA and DHCA at all the concentrations studied (0.1, 1, 10, and 20 μM) and DHFA at 10 μM after 16 h incubation up to 24 hours showed a significant decrease in the production of ROS compared to the control. Only YME at 50 $\mu\text{g/mL}$ significantly increased ROS generation.

With respect to the antioxidant defense system, there were no statistically significant changes in GSH levels either in the activity of antioxidant enzymes GPx and GR. Accordingly, there were no statistical differences in protein oxidation after 20 h direct treatment with the extracts, phenolic compound and metabolites as shown by the levels of carbonyl groups (Table 1). Thus, it can be assumed that the range of concentration tested can be safely used to study the protective effect of YME, GCBE, 5-CQA, DHCA, and DHFA against a condition of oxidative stress in endothelial cells. Interestingly, after the treatment with the different compounds and extracts for 20 h, eNOS levels were similar to the controls, except with GCBE at 20 and 50 $\mu\text{g/mL}$ which augmented significantly.

Protective effects of YME, GCBE, 5-CQA, DHCA and DHFA in a condition of oxidative stress and inflammation in EA.hy926 cells

Induction of oxidative stress in EA.hy926 cells by TNF- α

The concentrations of TNF- α tested were selected attending to the literature: 5 ng/mL in 3T3-L1 pre-adipocytes, 10 ng/mL in HUVEC cells (Wang et al, 2014), EA.hy936 cells (Gomez-Juaristi et al, 2018⁶; Yang et al, 2014), Caco-2 cells, and in HBMEC cells, and 20 and 40 ng/mL TNF- α in Caco-2 cells (Rodriguez-Ramiro et al, 2013).⁴⁷ Afterwards, an experiment was carried out in EA.hy926 cells to measure the effects of 5, 10, 20, and 40 ng/mL TNF- α for 0.5, 1, 2, 4, 20 and 24 h on ROS production (Fig. 1). After 20 h treatment, 5, 10, 20 and 40 ng/mL TNF- α induced a sharp increase in ROS production, which was even higher at 24 h. Attending to these results, TNF- α at 10 ng/mL for 24 h was the condition selected to induce oxidative stress in the present work and to evaluate the protective effects of YME, GCBE, 5-

CQA, DHCA, and DHFA in EA.hy926 cells against this condition. In these experiments, a negative control, cells non-treated with TNF- α , and a positive control, cells treated with TNF- α at 10 ng/mL for 24 h but not exposed to the extracts, phenolic compounds or metabolites, were used.

Other experiments were carried out in EA.hy926 cells in order to evaluate the effects of different concentrations of TNF- α and incubation times on the concentration of eNOS. Fig. 2A shows that TNF- α significantly decreased eNOS levels from 10 ng/mL to 40 ng/mL compared to control cells after 24h treatment, whereas Fig. 2B shows that exposure to 10 ng/mL TNF- α from 4 till 24 hours reduced eNOS levels compared to the control, showing the lowest levels after 24 h. Attending to these outcomes, the treatment conditions aforementioned were considered adequate to evaluate the possible protective effects of the extracts, phenolic compound and metabolites on eNOS concentration.

Cell cytotoxicity

Treatment with TNF- α at 10 ng/mL after 24 h significantly enhanced (1.4 times) LDH leakage compared with the negative control, indicating prominent cell damage in EA.hy926 cells (Fig. 3). However, when the cells were pre-treated with YME and GCBE at 20 and 50 μ g/mL, a significant decrease in the cytotoxicity occurred, whereas with 1 μ g/mL of both extracts the decrease was small and not statistically relevant. However, 5-CQA at all the concentrations tested (0.1, 1, 10, and 20 μ M) and DHCA at 0.3, 1, and 10 μ M significantly decreased the cytotoxicity induced by TNF- α compared with the stressed control, in contrast to DHFA that did not reduce LDH levels (Fig. 3).

ROS generation

EA.hy926 cells treated with the pro-inflammatory molecule for 24 h showed an approximate 40% increase in intracellular ROS generation compared to non-stressed controls (Fig. 4). Pre-treatment with YME and GCBE at 20 and 50 µg/mL, 5-CQA at 1, 10, and 20 µM, and DHCA at 0.3, 1, and 10 µM significantly decreased ROS production compared to the TNF- α control, i.e. the lower concentrations of test compounds did not induce significant reductions. Similarly to in the previous experiment and Baeza et al. (2016a, 2016b), DHFA failed to protect the cells from the pro-oxidant effect of TNF- α .

Antioxidant defenses

Non-enzymatic defenses: TNF- α in EA.hy926 cells evoked around a 30% decrease in the steady-state concentration of GSH (Fig. 5). Pre-treatment with 20 and 50 µg/mL YME, 50 µg/mL GCBE, 1-20 µM 5-CQA, and 1-10 µM DHCA significantly increased GSH levels, partly recovering the TNF- α -induced depleted concentrations. DHFA only afforded a partial recovery of GSH levels at the highest (10 µM) dose tested (Fig. 6D).

Enzymatic defenses: The enzyme activities of GPx and GR increased about 30 and 90%, respectively after incubation with 10 ng/mL TNF- α in EA.hy926 cells, indicating a positive response of the cell defense system to the pro-inflammatory challenge (Fig. 6 and 7). When cells were pre-treated with 20-50 µg/mL YME and GCBE, 10-20 µM 5-CQA, and 10 µM DHCA for 20 h, the chemically-induced rise in GPx activity was prevented compared with stressed cells, whilst DHFA had no effect.

In line with GPx results, GR over-activation was also restricted compared with TNF- α treated cells after pre-incubation of EA.hy926 endothelial cells with 1-50 µg/mL

YME, 20-50 µg/mL GCBE, 1-20 µM 5-CQA, and 0.3-10 µM DHCA, while only the highest dose (10 µM) of DHFA showed some effect on GR activity (Fig. 8). Lower concentrations of YME, GCBE, 5-CQA, and DHCA did not significantly decrease GPx and GR activities (Fig. 6 and 7).

Biomarker of protein oxidative damage

Endothelial EA.hy926 cells treated with 10 ng/mL TNF- α for 24 h showed a significant increase in the cellular concentration of carbonyl groups, indicating oxidative damage to cell proteins (Fig. 8). However, pre-treatment of EA.hy926 cells with 1-50 µg/mL YME and GCBE, 1-20 µM 5-CQA, and 0.3-10 µM DHCA and 1-10 µM DHFA for 20 h significantly reduced the level of carbonyl groups in response to TNF- α , indicating a reduced level of protein oxidation in response to the stressor.

Concentration of endothelial nitric oxide synthase

The effects of the treatment with YME, GCBE, 5-CQA, DHCA and DHFA, at the indicated concentrations on the levels of eNOS were also investigated in TNF- α stimulated EA.hy926 cells. Conditions of 1-50 µg/mL of YME and GCBE, as well as 1-20 µM 5-CQA increased eNOS concentration in a dose dependent manner, recovering the levels of this enzyme depleted by TNF- α exposure. In contrast, DHCA and DHFA increased eNOS levels similarly at all the concentrations tested, even at the lowest dose (0.1 µM, Fig. 9).

DISCUSSION

The consumption of yerba mate and coffee, mainly due to the rich content in CGAs of the beverages, has been reported to reduce the risk of chronic diseases such as cardiovascular disease (van Dam & Feskens, 2002; Ding et al, 2014; de Moraes Pontilho et al, 2015). It is well known that the bioactivity of CGAs depends on its bioavailability which is largely dependent on its metabolism by microorganisms in the gut (Olthof et al, 2001; Gonthier et al, 2003). In human bioavailability studies carried out in our group using yerba mate (Gómez-Juaristi et al. 2018b), it was confirmed that DHFA and DHCA are among the most relevant CGA metabolites observed in plasma reaching maximum concentrations (0.1 and 0.3 mM) 8h the intake of the beverages (Gomez-Juaristi et al, 2018a; 2018b; Lang et al, 2013). In two *in vitro* studies recently carried out in our group, a yerba mate and green bean phenolic extract showed high capacity to protect human HepG2 cells against oxidative stress induced by *Tert*-butylhydroperoxide being remarkable the protective effect of DHCA, and to a lower extent that of DHFA, even at low, physiological concentrations (Baeza et al, 2016a,2016b). In another study, also 5-CQA and the other main hydroxycinnamic acid in coffee, 3,5-dicaffeoylquinic acid, presented significant protection against oxidative stress using the same cell model (Baeza et al, 2014), in agreement with other research groups that described antioxidant effects of 5-CQA in PC-12 cells (Park, 2013).

Before investigating the endothelial-protective effects of the extracts, 5-CQA, DHCA, and DHFA, it was crucial to evaluate their cellular safety at the concentrations tested. None of the doses of YME, GCBE, 5-CQA, DHCA, and DHFA induced direct cell damage or cytotoxic effects (Table 1). YME at 20 µg/mL, GCBE at 20 and 50 µg/mL, 5-CQA at 0.1, 1, 10, and 20 µM, DHCA at 0.3, 1 and 10 µM, and DHFA at 10 µM significantly reduced ROS generation (Table 2). In contrast, YME at 50 µg/mL

increased ROS production. Nevertheless, the YME concentration of 50 µg/mL was further studied considering that 100 µg/mL has been described as the threshold for YME to produce negative effects (Muñoz-Culla et al, 2016). Absence of a direct effect of the compounds studied on antioxidant defenses was observed, as GSH, GPx and GR values were not altered, and neither were the levels of carbonyl groups as biomarkers of protein oxidation (Table 1). Therefore, all the concentrations studied of the extracts, phenolic compound and metabolites, except YME at 50 µg/mL, were favorable conditions for the endothelial cells to face an oxidative stress challenge. Accordingly, the studied compounds did not change, or slightly enhanced, the levels of eNOS, except GCBE at 20 and 50 µg/mL, which significantly increased the enzyme concentration.

TNF- α induced oxidative stress conditions in the vascular cells used, in agreement with previous studies (Chen et al, 2009; Granado-Serrano et al, 2012). Before starting the study, different TNF- α concentrations and incubation times were tested to set up an adequate oxidative stress model using EA.hy926 endothelial cells which also allowed looking into endothelial dysfunction. Although multiple mechanisms are involved in endothelial cell injury, increased oxidative stress seems to be the common underlying mechanism for the development of endothelial dysfunction. Considering that ROS production is a good indicator of the oxidative status of cells (Alía et al, 2005), we tested the production of ROS induced by TNF- α from 5-40 ng/mL. TNF- α from 5 ng/mL after 20 h incubation produced a significant increase in ROS production (Fig. 1). In addition, TNF- α , from 10 ng/mL caused a reduction in eNOS concentration from incubation times of 4 hours up to 24 hours (Fig. 2). Therefore, we used TNF- α at 10 ng/mL and an exposure time of 24 h to assure an oxidative stress and endothelial dysfunction condition. The selected concentration and incubation time have already been applied in previous oxidative stress studies

(Granado-Serrano et al, 2012; Rodriguez-Ramiro et al, 2013) knowing that the indicated concentration is above the healthy physiological range (14.2–61.7 pg/mL; Kokkonen et al, 2010).

Treatment of endothelial cells with TNF- α at the aforementioned conditions, lead to high LDH leakage. However, pre-treatment with YME, GCBE, 5-CQA, and DHCA exhibited a significant decrease in cytotoxicity, except for DHFA which showed no effect (Fig. 3). Similarly, TNF- α -induced increase in ROS generation was efficiently quenched when the cells were pre-treated with the studied extracts and phenolic compound. Regarding the metabolites, DHCA was as, or even more, effective than the parent compound (5-CQA) decreasing ROS generation in TNF- α -challenged cells from 0.1 μ M, whilst DHFA did not reduce ROS levels (Fig. 4).

Regarding GSH levels, it is usually assumed that GSH depletion reflects intracellular oxidation, whereas a balanced GSH concentration is expected to prepare the cells against a potential oxidative insult (Martin et al, 2014; Lee & Lee, 2008).^{43,48} In the present study, treatment with TNF- α induced a significant decrease in intracellular GSH levels; however, pre-treatment with YME and GCBE, 5-CQA, and DHCA partly prevented the depletion of GSH (Fig. 5), in agreement with other studies using drugs such as tempol (Mariappan et al 2007), or a cocoa phenolic extract and its main phenolic compounds (epicatechin and procyanidin B2; Rodriguez-Ramiro et al 2011). TNF- α treatment also dramatically affected antioxidant enzymes in EA.hy926 cells, evoking a significant increase of GPx (Fig. 6) and GR (Fig. 7) activities to cope with ROS increase and thus pointing to a positive response of the endothelial cell defense system to face the oxidative insult. However, cells pre-treated with YME, GCBE, 5-CQA and DHCA, although not at the lowest concentration, reduced GPx (Fig. 6) activity to values similar to the control whereas DHFA did not produce changes.

Similarly, the studied compounds reduced GR activity except for DHFA (Fig. 7). Similar protective effects have also been described for phenolics in cocoa (Martín et al, 2013) green coffee beans (Baeza et al, 2014) and grape pomace extracts (Wang et al, 2016) in INS-1E, HepG2 and Caco-2 cells, respectively, as well as for thistle (*Silybum marianum*) in EA.hy926 cells (Palomino et al., 2017) using other oxidative stressors. Gathering the direct and indirect results observed in the present study, they are in accordance with other studies, using phenolic extracts from *Corema album* (León-González et al, 2012) or cranberry (Martín et al, 2015) rich in hydroxycinnamic acids, which had no patent effect on steady-state levels of GSH and activity of GPx and GR but showed significant chemo-protective activity against a challenge with a potent pro-oxidant. It may be concluded that certain bioactive compounds, or combinations, do not alter the basal redox status but can protect the antioxidant defense system in the context of an oxidative/inflammatory stress situation. Additionally, the potent *in vitro* antioxidant capacity of 5-CQA should not be ruled out (Baeza et al, 2014).

Carbonyl groups are considered a consistent biomarker of oxidative damage to proteins, because of its relatively early formation and the chemical stability of oxidized proteins (Dalle-Donne et al, 2003). Pre-treatment with all the tested compounds significantly reduced the levels of carbonyl groups (Fig. 8), similarly to epicatechin (Martín et al, 2010) among other phenols, in HepG2 cells, and cocoa epicatechin in the pancreatic beta cells (Martín et al, 2014).

As aforementioned, eNOS is the main factor responsible for vasodilation and maintenance of vascular tone, among other anti-atherogenic effects (Andriantsitohaina et al, 2012). To different degrees, a red wine phenolic extract (Leikert et al, 2002) cyanidin, *p*-coumaric acid, caffeic acid, benzoic acid, and vanillic acid (Wallerath et al, 2005) have shown to enhance eNOS expression, as well as resveratrol metabolites

(Ladurner et al, 2014). Conversely, quercetin produced null or even negative effects on eNOS expression (Huisman et al, 2004; Tribolo et al, 2013). However, to our knowledge, this is the first study that evaluates the effects of YME, GCBE, CGA, DHCA, and DHFA on eNOS levels in endothelial cells. We observed a dose-dependent increase with both extracts and 5-CQA after TNF- α induced stress. Regarding the metabolites, it is noteworthy that there was also a significant increase in eNOS levels from the lowest concentrations, pointing to these metabolites having potent effects against endothelial dysfunction.

As discussed above, most of the *in vitro* studies on the potential effects of polyphenol-rich products or their main phenolic compounds have been performed with high doses of extracts or pure polyphenols, often overlooking the low bioavailability of phenolic compounds and the major metabolism undergone either by phase II conjugating enzymes, yielding methylated, sulphated and/or glucuronidated metabolites, or by the gut microbiota (Gómez-Juaristi et al, 2018a; Bravo, 1998). In the present study, we used physiologically relevant doses of YME, GCBE, and 5-CQA, as well as DHCA and DHFA, the two major microbial metabolites identified in plasma and urine samples after consumption of CGAs-rich foods. We used doses of these metabolites that had been found in plasma in healthy individuals after consuming a single serving of yerba mate tea (Gómez-Juaristi et al, 2018a) and green/roasted coffee (Gómez-Juaristi et al, 2018a), which confers relevance to the results here reported, showing that specially DHCA can have important protective effects on endothelial cells at concentrations naturally found in circulation.

An interesting finding in the present work was that the microbial metabolite DHCA shows relevant antioxidant and endothelial protective properties, whereas DHFA was less effective. The difference between both molecules is the methylation of

the hydroxyl group of the phenolic ring in DHFA, which suggests the importance of the ortho-diphenolic group in the biological activity of polyphenols (Bravo, 1998; Huang et al, 2005). The endothelial protection afforded by YME, GCBE and their major phenolic constituent and circulating metabolites could contribute to the health benefits of these beverages including a possible protective effect against CVD. It is noteworthy that microbial phenolic metabolites may independently present bioactivity improving vascular function, pointing to being probably the main active compounds *in vivo*. The molecular mechanisms involved in the observed effects of the metabolites deserve to be studied. Similarly, these extracts, phenolic compounds and metabolites have also shown to be effective inhibitors of *in vitro* platelet activation, decreasing adenosine 5-diphosphate (ADP)-induced P-selectin expression in human platelets, thus contributing to a potential cardio-protective effect of yerba mate and green coffee (Baeza et al, 2017).

CONCLUSION

Yerba mate and green coffee bean extracts showed similar protective effects against oxidation and endothelial dysfunction induced by TNF- α in EA.hy926 cells, with the major phenolic compound of both extracts (5-CQA) clearly contributing to such protective effect. One of the major 5-CQA microbial metabolites, DHCA, is likely to play an important role in the protection due to its antioxidant activity, whereas the other 5-CQA relevant microbial metabolite studied, DHFA, showed lower activity. Moreover, the extracts, 5-CQA, DHCA and DHFA increased the production of eNOS.

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FIGURE CAPTIONS

Fig. 1. Effect of TNF- α on reactive oxygen species (ROS) production in human umbilical cord endothelial EA.hy926 cells. Values are means and the vertical bars are standard deviations. Cells were treated with 5, 10, 20, and 40 ng/mL tumor necrosis factor-alpha (TNF- α) for 0.5, 1, 2, 4, 20, and 24 h and ROS production was measured. Results are means $\pm$ SD (n = 6-8). Different letters denote statistically significant differences (p<0.05).

Fig. 2. Effect of tumour necrosis factor-alpha (TNF- α) on eNOS in human umbilical cord endothelial EA.hy926 cells. Values are means with standard deviations represented by vertical bars. (A) Cells were treated with 10, 20, and 40 ng/mL TNF- α for 24 h. (B) Cells were treated with 10 ng/mL TNF- α for 4, 6, 8, and 24 h. Results are means $\pm$ SD (n = 6-8). Different letters denote statistically significant differences (p<0.05).

Fig. 3. Protective effect of pre-treatment with yerba mate extract (YME) and green coffee bean extract (GCBE, A), 5-caffeoylquinic acid (5-CQA, B), dihydrocaffeic acid (DHCA, C), and dihydroferulic acid (DHFA, D) for 20 h on cell toxicity (%LDH) induced by tumour necrosis factor-alpha (TNF- α) for 24 h. Results are means $\pm$ SD (n = 6-8). Different letters denote statistically significant differences (p<0.05).

Fig. 4. Protective effect on intracellular ROS production by human umbilical cord endothelial EA.hy926 cells pre-treated with the noted concentrations of yerba mate extract (YME, A), green coffee bean extract (GCBE, B), 5-caffeoylquinic acid (5-CQA, C), dihydrocaffeic acid (DHCA, D), and dihydroferulic acid (DHFA, E), and after tumour necrosis factor-alpha (TNF- α) exposure for 24 h. Results are expressed as % of fluorescence arbitrary units against time (n = 4-6). Different letters within the same time denote statistically significant differences (p<0.05).

Fig. 5. Protective effect of pre-treatment with yerba mate extract (YME), green coffee bean extract (GCBE, A), 5-caffeoylquinic acid (5-CQA, B), dihydrocaffeic acid (DHCA, C), and dihydroferulic acid (DHFA, D) for 20 h, and after tumour necrosis factor-alpha (TNF- α) exposure for 24 h on GSH levels in human umbilical cord endothelial EA.hy926 cells. Results are means $\pm$ SD (n = 6-8). Different letters denote statistically significant differences (p<0.05).

Fig. 6. Protective effect of pre-treatment with yerba mate extract (YME), green coffee bean extract (GCBE, A), 5-caffeoylquinic acid (5-CQA, B), dihydrocaffeic acid (DHCA, C), and dihydroferulic acid (DHFA, D) for 20 h, and after tumour necrosis factor-alpha (TNF- α) oxidative insult for 24 h on human umbilical cord endothelial EA.hy926 cell antioxidant enzyme GPx. Results are means $\pm$ SD (n = 6-8). Different letters denote statistically significant differences (p<0.05).

Fig. 7. Protective effect of pre-treatment with yerba mate extract (YME), green coffee bean extract (GCBE, A), 5-caffeoylquinic acid (5-CQA, B), dihydrocaffeic acid (DHCA, C), and dihydroferulic acid (DHFA, D) for 20 h, and after tumour necrosis factor-alpha (TNF- α) oxidative insult for 24 h on human umbilical cord endothelial EA.hy926 cell antioxidant enzyme GR. Results are means $\pm$ SD (n = 6-8). Different letters denote statistically significant differences (p<0.05).

Fig. 8. Protective effect of pre-treatment with yerba mate extract (YME), green coffee bean extract (GCBE, A), 5-caffeoylquinic acid (5-CQA, B), dihydrocaffeic acid (DHCA, C), and dihydroferulic acid (DHFA, D) against oxidative stress induced by tumour necrosis factor-alpha (TNF- α) for 24 h on human umbilical cord endothelial

EA.hy926 cell protein-oxidation (carbonyl groups). Results are means $\pm$ SD (n = 6-8).

Different letters denote statistically significant differences ($p < 0.05$).

Fig. 9. Protective effect of pre-treatment with yerba mate extract (YME, A), green coffee bean extract (GCBE, B), 5-caffeoylquinic acid (5-CQA, C), dihydrocaffeic acid (DHCA, D), and dihydroferulic acid (DHFA, E) against oxidative stress induced by tumour necrosis factor-alpha (TNF- α) for 24 h on the endothelial nitric oxide synthase (eNOS) levels in human umbilical cord endothelial EA.hy926 cells. Results are means $\pm$ SD (n = 6-8). Different letters denote statistically significant differences ($p < 0.05$).