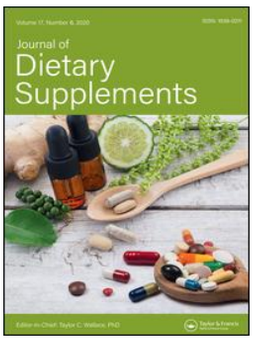

Journal of Dietary Supplements

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/ijds20

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To cite this article: Aparna Shil, Chris Davies, Lata Gautam, Justin Roberts & Havovi Chichger (2021): Investigating the Opposing Effect of Two Different Green Tea Supplements on Oxidative Stress, Mitochondrial Function and Cell Viability in HepG2 Cells, Journal of Dietary Supplements, DOI: 10.1080/19390211.2021.1894304

To link to this article: https://doi.org/10.1080/19390211.2021.1894304

Published online: 17 Mar 2021.

JOURNAL OF DIETARY SUPPLEMENTS
https://doi.org/10.1080/19390211.2021.1894304

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Investigating the Opposing Effect of Two Different Green Tea Supplements on Oxidative Stress, Mitochondrial Function and Cell Viability in HepG2 Cells

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ABSTRACT

Green tea extract (GTE) improves exercise outcomes and reduces obesity. However, case studies indicate contradictory physiology regarding liver function and toxicity. We studied the effect of two different decaffeinated GTE (dGTE) products, from a non-commercial (dGTE1) and commercial (dGTE2) supplier, on hepatocyte function using the human cell model, HepG2. dGTE1 was protective against hydrogen peroxide (H_2O_2)-induced apoptosis and cell death by attenuating oxidative stress pathways. Conversely, dGTE2 increased cellular and mitochondrial oxidative stress and apoptosis. A bioavailability study with dGTE showed the major catechin in GTE, EGCG, reached 0.263 $\mu g \cdot ml^{-1}$. *In vitro*, at this concentration, EGCG mimicked the protective effect of dGTE1. GC/MS analysis identified steric acid and higher levels of palmitic acid in dGTE2 versus dGTE1 supplements. We demonstrate the significant biological differences between two GTE supplements which may have potential implications for manufacturers and consumers to be aware of the biological effects of supplementation.

KEYWORDS

Basic science research; cell biology; green tea extract; hepatic cells; oxidative stress

Introduction

Green tea is an unfermented, commonly-consumed beverage which has been extensively studied as a natural remedy with potential medicinal and physiological health benefits. Indeed, green tea extract (GTE), is listed in more than 100 over-the-counter herbal preparations, largely due to the range of polyphenols present in the extract (Green 2012). In particular, GTE is rich in polyphenol compounds or catechins, with four main catechins identified in GTE: (-)-epigallocatechin-3-gallate (EGCG), (-)-epicatechin-3-gallate, (-)-epigallocatechin (EGC), and (-)-epicatechin). EGCG is the most abundant and pharmacologically-active of the catechins present in GTE (Henning et al. 2003). Although caffeine is present in GTE, research has demonstrated that the benefits of

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GTE supplementation are not specific to the caffeine content with many studies using the decaffeinated form of the extract (Dulloo et al. 1996; Roberts et al. 2015).

In recent years, green tea extract has been extensively studied due to its role in reducing obesity. In a range of *in vivo* models employing high-fat dietary strategies, GTE supplementation has been established to affect adipocytes by inhibiting adipogenesis and lipogenesis and activating fat browning to reduce obesity and hypertriglyceridemia (Neyrinck et al. 2017; Pan et al. 2017; Cho et al. 2019). Interestingly, this has been linked with improved insulin sensitivity and increased GLUT4 expression in adipocytes associated with an increase in activity of the ubiquitous signaling molecule, AMP-activated protein kinase (AMPK) (Rocha et al. 2016). Following GTE supplementation, mice fed a high-fat diet coupled with voluntary exercise demonstrate significantly reduced body weight, total visceral fat and plasma insulin, linked to increased expression of PPAR-γ coactivator 1α and various members of the mitochondrial electron chain (Sae-Tan et al. 2014). In human studies, GTE consumption has been demonstrated to increase fat oxidation in response to intermittent sprinting exercise and moderate-intensity exercise, and to improve exercise performance (Venables et al. 2008; Gahreman et al. 2015; Roberts et al. 2015). Thus GTE supplementation has been studied extensively with respect to improved exercise capacity, enhanced metabolic flexibility and reduced obesity.

From this protective effect on obesity and hypertriglyceridemia, it is likely that GTE supplementation will reduce hepatic steatosis in settings of obesity. However, there is controversy in the literature regarding the impact of GTE on the liver. There are several studies which indicate the benefit of GTE with regards to health and disease. GTE supplementation in mice fed a high-fat diet caused a significant reduction in body weight, hepatic fat accumulation, hypertriglyceridemia along with improved insulin resistance (Bae et al. 2018). There are also studies which demonstrate that GTE protects against liver injury induced by a range of environmental factors including bacterial endotoxin, lead, alcohol, acetaminophen and D-galactosamine (He et al. 2001; Kager et al. 2010; Mehana et al. 2012; Park et al. 2012; Lu et al. 2013). This hepatoprotective role was noted alongside reduced levels of alanine aminotransferase (ALT) and has been implicated as a result of an anti-inflammatory function of GTE, with reduced TNF-alpha levels and NF-Kappa B signaling observed in various models of liver injury (He et al. 2001; Lin et al. 2009). The antioxidant role of the polyphenol has also been investigated, with reduced oxidative stress, ROS accumulation and DNA damage occurring in hepatocytes following GTE supplementation (Lin et al. 2009; Kager et al. 2010). Conversely, there are studies which indicate GTE-induced hepatotoxicity in animals. Emoto *et al* demonstrate that GTE administration caused increased mortality in rats associated with hepatocellular hypertrophy, necrosis, and inflammation (Bae et al. 2018). Furthermore, individual case studies in humans are suggestive of potential hepatotoxicity with an increase in plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin, as well as specific impact such as hepatic fibrosis and inflammation (Molinari et al. 2006; Mazzanti et al. 2009; Green 2012; Patel et al. 2013). More recently, the United State Pharmacopeia reviewed hepatotoxicity of GTE and noted that clinical studies and case reports suggest a link between the supplement and declining liver function (Oketch-Rabah et al. 2020). There are, however, several confounding

factors in these *in vivo* and human studies, such as a non-physiological mode of administration (for example, intraperitoneal injection), an elderly population (for example, 60-80 years), predisposing hepatic conditions, longevity and dose of GTE consumption (e.g. $>800\,mg{\cdot}d^{-1}$) and composition of GTE supplementation (for example, caffeinated, sweetened). Whilst these factors do not diminish the potential hepatotoxic effects of GTE supplementation, they indicate the need to further study and understand the molecular mechanisms linking GTE and hepatocyte function.

There are few *in vitro* hepatocyte studies to understand whether the supplement is cytoprotective or cytotoxic. Seo *et al* demonstrate a significant role for GTE in reducing hepatocyte migration, but no impact on cytotoxicity (Seo et al. 2016). The majority of *in vitro* studies are performed with a major component of GTE, EGCG. Several studies demonstrate that EGCG reduces hepatocyte viability due to reduced proliferation and increased apoptosis but with no impact on mitochondrial function (Schmidt et al. 2005; Mezera et al. 2016; Sabry et al. 2019). Other studies indicate that EGCG promotes autophagy and cell survival and protects against cellular and mitochondrial oxidative stress induced by glucosamine (Mi et al. 2017; Zhao et al. 2017). There is therefore conflicting data surrounding *in vitro* studies with both GTE and EGCG, however it is worth noting that these studies use a range of EGCG concentrations which are much higher than physiologically-relevant (Krupkova et al. 2016; Liu et al. 2018). Therefore there is controversy in these *in vitro* studies too, with few studies addressing the discrepancy between these studies and dissecting the mechanism of GTE action on hepatocytes.

In the present study, we evaluate the biological effect of two different decaffeinated GTE (dGTE) products, with similar levels of EGCG, on a human hepatocyte *in vitro* model. We utilized a dGTE compound directly from source, i.e. non-commercial (dGTE1) and a dGTE which is commercially-available (dGTE2). We demonstrate that the two dGTEs display opposing effects on HepG2 cell viability with dGTE2 increasing cell death through apoptosis whilst dGTE1 protects against hydrogen-peroxide induced cell death. These opposing effects are also seen in markers of oxidative stress and mitochondrial dysfunction and were linked to differing chemical compositions of the two dGTE studied. The studies presented here demonstrate the biological and chemical differences in two different compounds which are supposedly the same supplement. This raises questions for manufacturers and consumers, as well as researchers in the field, regarding how one type of supplement can have opposing effects.

Materials and methods

Cell lines and reagents

Human hepatocellular carcinoma cells (HepG2) were purchased from Sigma-Aldrich (Dorset, UK), cultured in Dulbecco's Minimum Essential Media containing 10% fetal bovine serum, 1% glutamine and 1% penicillin/streptomycin and used between passage 35-50. DCFDA, glutathione (ab65322), catalase (ab83464) and superoxide dismutase (ab65354) activity assay kits were purchased from Abcam (Cambridge, UK). JC-1 and MitoSox^{TM} dye was purchased from ThermoFisher Scientific (Hemel Hempstead, UK). Actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Annexin V kit was purchased from BD Pharmingen (Wokingham, UK) and the GSH

Bioxytech kit, phosphorylated (Thr172) and unphosphorylated AMPK antibodies were obtained from Merck Millipore (Hertfordshire, UK). The AMPK activity assay kit was purchased from MBL International (Buckingham, UK). Green tea extract 1 (dGTE1) was sourced from a food ingredient manufacturer whilst green tea extract 2 (dGTE2) was sourced directly from a supplement company as previously described (Roberts et al. 2015). For confidentiality, brand names have not been included. All other reagents were purchased from Sigma Aldrich (Dorset, UK).

Preparation of the HepG2 cell treatments

Two types of decaffeinated green tea extract were studied: dGTE1 was provided as an anhydrous capsulated powder standardized to 70% for EGCG such that each capsule provided a total of 571 mg dGTE, of which 400 mg was EGCG. dGTE2 was also provided as an anhydrous capsulated powder standardized to 98% polyphenols such that each capsule provided a total of 725 mg of GTE, of which 326.25 mg was EGCG. In addition, (-)-Epigallocatechin gallate (EGCG) was used for experiments studying ROS, viability, apoptosis, AMPK and mitochondrial function. dGTE1, dGTE2 and EGCG were prepared as a 1 $mg.ml^{-1}$ stock in distilled water, warmed to 37 °C to aid solubilization, and sterile-filtered prior to use in cell culture.

Cells were exposed to dGTE1 or dGTE2 in the presence and absence of hydrogen peroxide (0.8 mM). Additional experiments were performed with a range of compounds, added at the time of GTE exposure, to assess mechanism. These compounds were: a) the pan-caspase inhibitor, Z-VAD-FMK (10 mM); b) the mitochondrial specific antioxidant MitoTEMPO (5 µM) (Figarola et al. 2015); c) Compound C (7.5 µM) pretreatment for 30 min prior to dGTE addition (Jeong et al. 2017); d) the antioxidant N-acetyl-cysteine (NAC) (10 mM). Unless otherwise stated, exposure was at the time of dGTE addition and for 24 h. A DMSO vehicle control was used for all compounds. In addition, HepG2 cells were cultured in serum-free (SF) media (0% BSA) for 24 h as a positive control for AMPK activity studies.

Annexin V assay

HepG2 cells were grown to 60% confluence in T-25 flasks prior to treatments for 6 h. Adhered and floating cells were collected and incubated with binding buffer, annexin V and propidium iodide for 15 mins in the dark. Cells were then analyzed with Accuri C6 Flow cytometer (BD Biosciences) and the percentage of positive cells for annexin V was calculated with FlowJo (V10.2, Oregon, USA) to give % apoptosis.

Cell viability studies

HepG2 cell viability was assessed using the Cell Counting Kit-8 (CCK-8). Cells were exposed to GTEs (range of concentrations), or compound treatments, for 24 h, followed by incubation with CCK-8 reagent for 2 h at 37 °C. Absorbance was then assessed at 450 nm using a microplate reader (Tecan Sunrise) and viability was calculated as % normalized to vehicle.

Western blot

HepG2 cells were exposed to dGTEs (concentrations ranging from 0.001 to 1000 μg.ml^{-1}), or compound treatments, for 24 h. Cells were then lysed with RIPA buffer, resuspended in Laemmli buffer and subjected to immunoblot analysis. Immunoblot analyses were performed on 10% SDS-PAGEs using a primary antibody specific to phosphorylated AMPK (Thr^{172}) or LKB1 (Ser^{428}), or caspase 3. Blots were stripped and reprobed for AMPK, LKB1 and β-actin. All antibodies were used at a dilution of 1:1000 and secondary antibody dilutions of 1:5000.

Cellular and mitochondrial ROS assay

Cellular ROS was assessed using the fluorogenic dye 2′,7′–dichlorofluorescin diacetate (DCFDA). HepG2 cells were plated on black-walled 96-well plate for 24 h followed by exposure to DCFDA (10 μM) for 30 min at 37 °C in the dark. DCFDA was then rinsed off the cells with PBS and replaced with dGTEs (range of concentrations), or compound treatments, for 2 h. DCFDA fluorescence was measured at 488 nm using a fluorescent plate reader (Victor, Perkin Elmer).

Mitochondrial ROS was assessed using the fluorogenic dye, MitoSox^{TM}. HepG2 cells were plated on black-walled 96-well plate for 24 h followed by treatment with dGTE1 and dGTE2 or compound treatments for a further 24 h. Cells were then washed with PBS and incubated with MitoSox^{TM} (5 μM) for 15 min at 37 °C. The fluorescence intensity was measured at 590 nm using a fluorescent plate reader (Victor, Perkin Elmer) (Figarola et al. 2015).

Determination of glutathione, catalase and superoxide dismutase activity, mitochondria membrane potential and AMPK activity levels

Cellular glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) activity, mitochondria membrane potential and AMPK activity levels were quantified using commercially-available kits. For all kits, HepG2 cells were plated in a 96-well plate and exposed to dGTEs (concentrations ranging from 0.001 to 1000 μg.ml^{-1}), or compound treatments, for 24 h. The assays were performed as per the manufacturer's protocol and absorbance was measured at 450 nm (SOD and AMPK activity) and 570 nm (CAT) using a microplate reader (Victor, Perkin Elmer). For the GSH activity kit, levels of the monochlorobimane dye bound to reduced or oxidized glutathione are quantified by fluorescence measured at an excitation and emission of 380/461 nm using a fluorescent plate reader (Victor, Perkin Elmer). For the JC-1 mitochondrial membrane potential assay, JC-1 fluorescence was measured at an excitation and emission of 520 nm and 590 nm using a fluorescent plate reader (Victor, Perkin Elmer). The change of mitochondrial membrane potential was measured as the ratio between aggregate (520 nm) and monomeric forms (590 nm) of JC-1. Increased ratios are indicative of mitochondrial membrane depolarization ($\Delta\Psi$m).

Caspase three activity assay

Caspase three activity was assessed in the presence and absence of dGTE1 and dGTE2. Cells were harvested into caspase-3 lysis buffer (10 mM HEPES pH 7.5, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM MgCl2, 5 mM EGTA, and pH adjusted to 7.5) at 4 °C. Collected cells were lysed using a series of three freeze-thaw cycles prior to centrifugation at 15,000 g for 10 min at 4 °C. 75 µg protein was then incubated with the reaction buffer (40 mM HEPES pH 7.5, 20% glycerol, and 4 mM DTT, 50 µM Ac-DEVD-AMC) at 37 °C for 1 h. Cleavage of Ac-DEVD-AMC was measured as pmol·mg^{-1}·min^{-1} using the N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin fluorogenic assay.

Bioavailability study

To investigate the bioavailability of dGTE, a prior pilot study was carried out with healthy volunteers. Following approval from the University of Hertfordshire, Life and Medical Sciences Ethics Committee (No. LS5/1/11P), 13 volunteers were recruited from personal contacts. All participants were required to be regularly engaged in physical exercise (>2 times per week), 18-25 years, in good general health (confirmed *via* a health screen questionnaire), with no known cardiovascular or metabolic abnormalities. Participants were required to have no known sensitivity to tea products, and not be consuming any nutritional supplements or medications which could conflict with the study parameters (e.g. caffeine, green tea extracts). All participants were required to provide written, informed consent prior to study inclusion.

Eleven participants (nine male, two female) took part in the study (M ± SD: age: 21 ± 1 years; height: 1.77 ± 0.09 cm; body mass: 75.07 ± 7.52 kg; body mass index (BMI): 23.9 ± 2.2 kg·m^2) and were instructed to refrain from consuming caffeinated and polyphenol-rich products (e.g. tea, coffee, chocolate, green vegetables, onions, fruit) for at least 24 h prior to each test visit. All testing took place at the University of Hertfordshire, Human Physiology Laboratory. For each visit, following a standardized overnight fast (∼12 h), participants were required to rest in a seated position for a venous whole blood sample collected into 4 ml K3EDTA Vacutainers (Greiner Bio-One GmbH, Kremsmunster, Austria) by a qualified phlebotomist. Samples were centrifuged for 10 mins at 2000 rpm, with plasma immediately frozen at −80 °C for later analysis. EGCG content of plasma was analyzed using liquid chromatography, tandem mass spectrometry (LC/MS/MS) by ABS Laboratories Ltd (BioPark, Welwyn Garden City) as previously described (de Lourdes Mata-Bilbao et al. 2007).

Following the initial blood sample, participants were fed a low polyphenol meal with 400 ml water, along with consumption of 571 mg capsulated dGTE1 or a lipid-coated control supplement. Further blood samples were collected at 2, 4 and 6 h post consumption. Trials were undertaken in a randomized, blinded manner.

GC-MS analysis of dGTE

dGTE1 and dGTE2 samples were prepared to 100 mg/ml concentration in methanol (100%) by vortex for 1 min. Undissolved content was separated by centrifugation at 14,000 rpm for 10 mins and 100 µl of the clear solvent layer was added to 1 ml of

Table 1. GC-MS parameters used for GTE sample analysis using the Shimadzu QP2010 instrument. *Panel a:* Parameters used for gas chromatography (GC); *Panel b:* Parameters used for mass spectrometry (MS).

a)	
Column specification	ZB-1, 30 m length, 0.25 mm diameter, 0.25 μm film thickness
Column programme	80 °C for 1 min, ramp at 15 °C·min^{-1} until 300 °C and hold for 5 mins
Injection port temperature	250 °C
Transfer line temperature	280 °C
Injection volume and mode	1 μL, 50:1 split mode
Carrier gas flow rate	Helium at 1 ml·min^{-1}

b)	
Ionization energy	70 eV
Solvent delay	2 mins
Scan range	50-500 amu
Ion source	70 eV, 230 °C

methanol in a fresh tube followed by repeat centrifugation. A 50 µl aliquot of the clear solvent layer was transferred to a glass vial, the solvent was evaporated and content reconstituted in 50 µl of the internal marker, leucomalachite green (LMG, 50 µg.ml^{-1}). This sample was analyzed using GC-MS using the Shimadzu QP2010 with the parameters in Table 1. Experiments were performed in triplicate, with sample preparation being repeated two times and two separate analyses performed, to ensure accuracy of readings. Identification of compounds was based on probability-matching of mass spectra using a computer library (NIST 2011, www.nist.gov, USA): results are to be intended as qualitative.

Statistical analysis

The experimental number is presented in the legend for each experiment. For two groups, the variance in data sets was analyzed using the Mann-Whitney test followed by the T-test. For three or more groups, variance was assessed by using Bartlett's test with data sets not reaching significance studied by Kruskal-Wallis test followed by Dunn's test. For all other data sets, differences among the means were tested for significance in all experiments by ANOVA with Tukey's range significance difference test or Bonferroni assessment (bioavailability study). Significance was reached when $p < 0.05$. Values are presented as mean ± standard error mean (S.E.M.).

Results

dGTE1 and dGTE2 display differential effects on hepatocyte viability and apoptosis

There are a wide range of concentrations which green tea extract are consumed by the general public, depending on the concentration available in the supplements provided. Our *in vitro* experiments were therefore to assess the effect of two dGTE compounds, dGTE1 (non-commercial) and dGTE2 (commercial), on liver cell function. To do this, we first measured HepG2 cell viability at a range of doses from sub-physiological (0.1 ng·ml^{-1}) to bioavailable (0.1 to 1 µg·ml^{-1}) to supra-physiological (1 mg·ml^{-1}) for

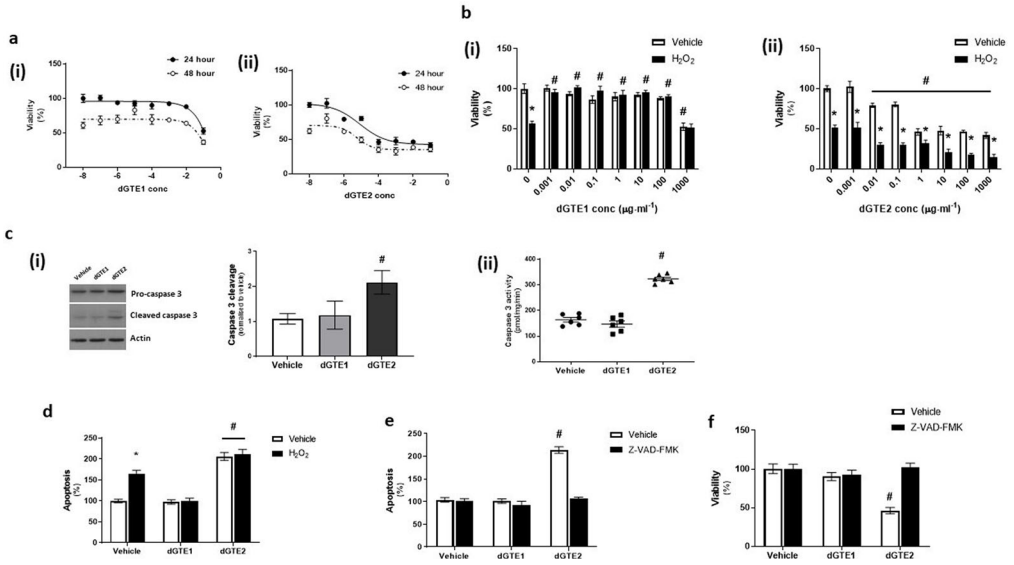

Figure 1. Green tea extract 1 and two display differential effects on hepatocyte viability and apoptosis. Panel a*:* HepG2 cells were exposed to varying concentrations of dGTE1 (i) or dGTE2 (ii) for time-points from 24 (closed symbol) and 48 h (open symbol). At the endpoint for each experiment, the CCK8 assay was performed to assess cell viability. *Panel b:* HepG2 cells were exposed to varying concentrations of dGTE1 (i) or dGTE2 (ii), or vehicle (H_2O), for 24 h in the presence (closed bars) and absence (open bars) of H_2O_2 (0.8 mM). Cell viability was measured by CCK8 assay. *Panel c:* Following exposure to dGTE1 and GTE2 (1 $\mu g \cdot ml^{-1}$), HepG2 cells were harvested and assessed by immunoblot (i) and N-acetyl-Asp-Glu-Val-Asp-7-amide-4-methylcoumarin fluorogenic assay (ii) for caspase three cleavage and activity respectively. A representative immunoblot is shown. *Panels d and e:* HepG2 were exposed to GTE1 or GTE2 (1 $\mu g \cdot ml^{-1}$), or vehicle (H_2O), for 6 h in the presence (closed bars) or absence (open bars) of H_2O_2 (0.8 mM) (d) or the pan-caspase inhibitor (Z-VAD-FMK) (e). Cells were exposed to annexin V and flow cytometry was used to assess % of cells which were undergoing apoptosis. *Panel f:* HepG2 were exposed to GTE1 or GTE2 (1 $\mu g \cdot ml^{-1}$), or vehicle (H_2O), for 24 h in the presence (closed bars) or absence (open bars) of Z-VAD-FMK and cell viability was assessed by CCK8 assay. Data are presented as mean ± SEM. $^{*}p < 0.05$ versus vehicle for H_2O_2, $^{\#}p < 0.05$ versus vehicle for dGTE.

both short (24 h) and long (48 h) time periods.dGTE1 was identified to be cytotoxic at concentrations exceeding 10 $\mu g \cdot ml^{-1}$, with EC_{50} value of 6729.8 $ng \cdot ml^{-1}$ for 24 h exposure studies (Figure 1a(i)). In contrast, dGTE2 was identified to be cytotoxic at concentrations exceeding 1 $ng \cdot ml^{-1}$ with an EC_{50} value of 8.2035 $ng \cdot ml^{-1}$ (Figure 1a(ii)). We next sought to establish the cytotoxic effect of each dGTE in settings of HepG2 cellular stress, using hydrogen peroxide (H_2O_2) as a model of cell injury. As previously demonstrated (Siddiqui et al. 2018), H_2O_2 exposure for 24 h significantly decreased cell viability (Figure 1b). When exposed to HepG2 cells concomitant with H_2O_2, dGTE1 exposure, from 0.001 to 100 $\mu g \cdot ml^{-1}$, was protective against H_2O_2-induced cell death (Figure 1b (i)) whilst dGTE2 exposure worsened H_2O_2-induced cell death at concentrations from 0.01 to 1000 $\mu g \cdot ml^{-1}$ (Figure 1b (ii)). Given these findings, further studies were performed using a concentration of 1 $\mu g \cdot ml^{-1}$ exposed to HepG2 to establish the mechanism through which dGTE1 and dGTE2 affect hepatocyte viability.

To assess whether the decrease in HepG2 cell viability was linked to apoptosis, we assessed activity of the pro-apoptotic regulator, caspase 3. Following exposure to

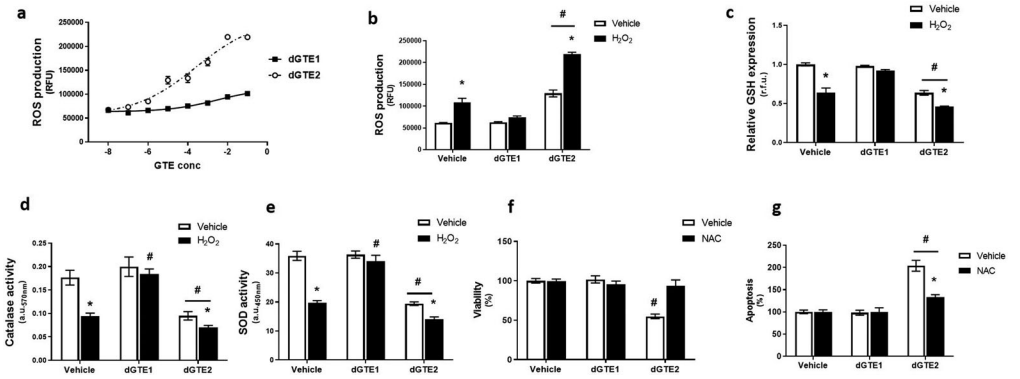

Figure 2. Green tea extract one attenuates hydrogen peroxide-induced ROS status whilst green tea extract two exacerbates oxidative stress to cause cytotoxicity. Panel a: HepG2 cells were exposed to varying concentrations of dGTE1 (square points) or dGTE2 (circle points) for 2 h. At the endpoint, ROS production was assessed as DCFDA incorporation. *Panels b-e:* ROS accumulation (b), GSH expression (c) and catalase (CAT) (d) or superoxide dismutase (SOD) (e) activity were assessed in HepG2 cells following exposure to dGTE1 and dGTE2 (1 $\mu g \cdot ml^{-1}$), or vehicle (H_2O), in the presence (closed bar) and absence (open bar) of H_2O_2 (0.8 mM) for 2 h. ROS accumulation was assessed by DCFDA incorporation, GSH expression was assessed using monochlorobimane dye, and CAT and SOD activity was assessed using colorimetric assay kits. *Panel f and g:* HepG2 cells were exposed to dGTE1 and dGTE2 (1 $\mu g \cdot ml^{-1}$), or vehicle (H_2O), in the presence (closed bar) and absence (open bar) of the antioxidant N-acetyl-cysteine (NAC) (10 mM). HepG2 cell viability (f) and apoptosis (e) were assessed using CCK8 and annexin V respectively. Data are presented as mean ± SEM. $^*p < 0.05$ versus vehicle for H_2O_2 or NAC, $\#p < 0.05$ versus vehicle for dGTE.

dGTE2, but not dGTE1, a significant increase in caspase-3 cleavage (Figure 1c (i)) and activity (Figure 1c (ii)) was observed which are indicative of apoptotic signals. Programmed cell death, through apoptosis, was then assessed using Annexin V to determine the % apoptotic cells in the population. Following exposure to H_2O_2 there was a significant increase in apoptosis which was attenuated by co-treatment with dGTE1, but not dGTE2 (Figure 1d). Furthermore, dGTE2, in the absence of H_2O_2 caused a significant increase in apoptosis which was attenuated by the pan-caspase inhibitor Z-VAD-FMK (Figure 1d and e), as was the decreased cell viability (Figure 1f). In contrast, dGTE1 exposure had no effect on % apoptosis in HepG2 cells and both apoptosis and viability were unaffected by Z-VAD-FMK (Figure 1d-f). Taken together these studies demonstrate that, at physiologically relevant concentrations, dGTE1 exhibits cytoprotective effects on HepG2 cells, whilst dGTE2 exhibit cytotoxic effects.

dGTE1 attenuates hydrogen peroxide-induced ROS status whilst dGTE2 exacerbates oxidative stress to cause cytotoxicity

We next sought to establish the mechanisms through which the two dGTEs exhibit these differing effects on HepG2 cells. As oxidative stress in hepatic epithelial cells has been closely linked to the efficacy of cytotoxic compounds and dGTE is associated with modulation of oxidative stress (Jayashree et al. 2017; Moon et al. 2018; Rodenak-Kladniew et al. 2018), we studied the impact of the two dGTEs on ROS accumulation. At increasing concentrations of dGTE1 and dGTE2, there was a dose-dependent increase in the production of intracellular ROS (Figure 2a). At the highest dGTE

Table 2. The effect of the antioxidant, NAC, on GSH, CAT and SOD activity and ROS accumulation in HepG2 cells. GSH, CAT, SOD activity and ROS accumulation were assessed in HepG2 following exposure to GTE1 or GTE2 in the presence and absence of the antioxidant N-acetyl-cysteine (NAC) for 24 h. Data are presented as mean ± SEM. $^{*}p < 0.05$ versus vehicle for NAC, $\#p < 0.05$ versus vehicle for GTE.

	Vehicle		dGTE1		dGTE2	
	Vehicle	NAC	Vehicle	NAC	Vehicle	NAC
Relative GSH (r.f.u.)	1.648 ± 0.158	1.937 ± 0.062	1.740 ± 0.174	1.876 ± 0.136	0.783 ± 0.052	1.588 ± 0.157*
CAT activity (a.u. $_{570\,nm}$)	0.177 ± 0.016	0.148 ± 0.086	0.183 ± 0.011	0.164 ± 0.017	0.095 ± 0.009#	0.12167 ± 0.008#*
SOD activity (a.u. $_{450\,nm}$)	35.9 ± 1.57	33.1 ± 1.66	36.4 ± 0.78	34.6 ± 1.86	19.4 ± 0.64#	29.0 ± 2.25#*
ROS production (r.f.u.)	63626 ± 1722	57983 ± 1618*	65215 ± 1977	59049 ± 1748*	122646 ± 11272#	64491 ± 3907*

concentration (100 μg·ml^{-1}) both extracts resulted in a significant increase in ROS accumulation however whilst there was a dramatic increase in ROS following exposure to dGTE2 (M ± SE: 230.02 ± 35.07%) there was only a moderate increase in ROS upon dGTE1 treatment (M ± SE: 54.29 ± 5.93%) (Figure 2a). Interestingly, at physiological concentrations of dGTE (0.1 to 1 μg·ml^{-1}), dGTE1 had no significant impact on oxidative stress whilst dGTE2 exerted a 2-fold increase in ROS accumulation (Figure 2a). Endogenous antioxidant enzymes, glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) are primarily responsible for clearing excess ROS and preventing oxidative stress (Balasubramaniyan et al. 2007).

To further study the effect of dGTE on oxidative stress, HepG2 cells were exposed to the oxidizing agent, H_2O_2, which significantly increases ROS production (Figure 2b) and significantly decreases GSH, CAT and SOD activity (Figures 2c-e). In dGTE1-treated cells, H_2O_2-induced ROS accumulation was blocked (Figure 2b), whilst GSH, CAT and SOD activity were elevated (Figure 2c-e). In contrast, H_2O_2-induced ROS accumulation was exacerbated by dGTE2 treatment (Figure 2b) alongside reduction in GSH, CAT and SOD activity (Figures 2c-e). To establish the link between ROS production and cytoprotective or cytotoxic effects of dGTE1 and dGTE2 respectively, the exogenous redox modulator and antioxidant, N-acetyl-cysteine (NAC) was utilized. NAC had no impact on dGTE1-treated cells but significantly regulated oxidative status of HepG2 cells by blocking dGTE2-induced oxidative stress as measured by GSH (% increase: 107.77 ± 24.49), CAT (% increase: 34.49 ± 16.05) and SOD activity (% increase: 49.17 ± 10.01), and ROS accumulation (% decrease: 46.61 ± 1.91) (Table 2). Interestingly, dGTE2-mediated reduction in viability (Figure 2f) through enhanced apoptosis (Figure 2g) was significantly attenuated by exposure with NAC. These data demonstrate that, at physiological concentrations, the two dGTEs exert opposing effects on intracellular ROS. These studies indicate that dGTE2 exerts a cytotoxic effect on HepG2 cells through elevated oxidative stress in HepG2 cells, whilst dGTE1 offers protective effects to reduce oxidative stress.

dGTE1 and dGTE2 differentially affect mitochondrial membrane potential and oxidative stress to regulate HepG2 cell viability

Given the role of dGTE1 and dGTE2 in regulating apoptosis and oxidative stress, and the importance of mitochondria in regulating ROS and cell fate (Michel et al. 2012), we

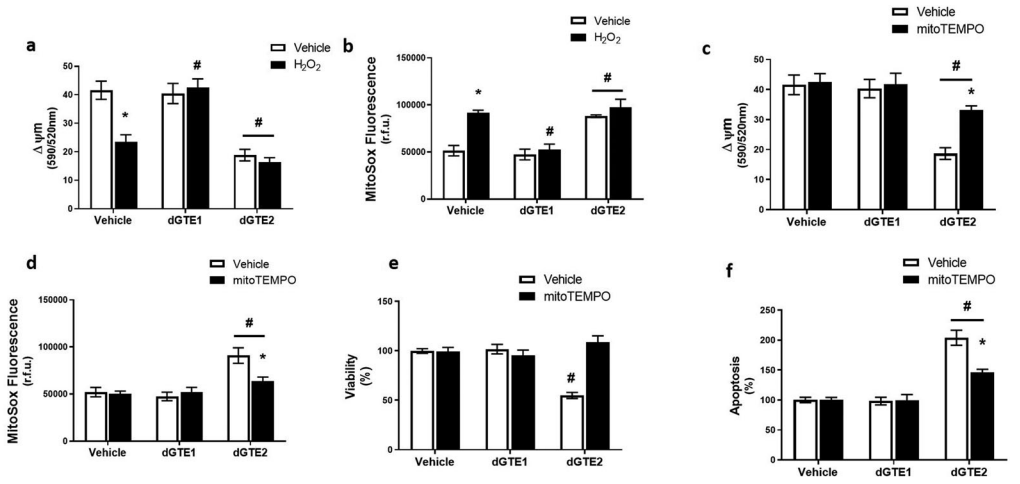

Figure 3. Green tea extract 1 and two differentially affect mitochondrial membrane potential and oxidative stress to regulate HepG2 cell viability. Panel a and b: HepG2 cells were exposed to dGTE1 and dGTE2 (1 μg·ml^{-1}), or vehicle (H_2O), in the presence (closed bar) and absence (open bar) of H_2O_2 (0.8 mM) for 2 h. Cells were then loaded with JC-1 dye (a) or MitoSOX^{TM} (b) for 10 mins to measure mitochondrial membrane potential (ΔΨm) or mitochondrial oxidative stress (mtROS), respectively, using fluorometry. Membrane depolarization was assessed by the 535/590 nm ratio (green/red monomers and aggregate). *Panel c-d:* HepG2 cells were exposed to dGTE1 and dGTE2 (1 μg·ml^{-1}), or vehicle (H_2O), in the presence (closed bar) and absence (open bar) of the mitochondrial antioxidant, mitoTEMPO (1 μM), for 2 h. ΔΨm (c) and mtROS (d) were then assessed using JC-1 dye or MitoSOX^{TM} respectively. *Panel e and f:* HepG2 cells were exposed to dGTE1 and dGTE2 (1 μg·ml^{-1}), or vehicle (H_2O), in the presence (closed bar) and absence (open bar) of mitoTEMPO (1 μM), for 24 h. HepG2 cell viability (e) and apoptosis (f) were assessed using CCK8 and annexin V respectively. Data are presented as mean ± SEM. $^*p < 0.05$ versus vehicle for H_2O_2 or mitoTEMPO, $\#p < 0.05$ versus vehicle for GTE.

next sought to understand the effect of these extracts on mitochondrial membrane potential (ΔΨm) and mitochondrial superoxide levels (mtROS), as assessed by the fluorescent dye JC-1 and MitoSox respectively (Figarola et al. 2015). Exposure to dGTE1 had no impact on either ΔΨm (Figure 3a) or mtROS levels (Figure 3b). In contrast, dGTE2 treatment significantly increased ΔΨm, and therefore membrane depolarization (Figure 3a), and mtROS levels (Figure 3b). Interestingly, dGTE1 significantly attenuated H_2O_2-induced membrane depolarization and mtROS formation, whilst dGTE2 exacerbated both (Figure 3a and b). Pretreatment with the mitochondria-specific antioxidant, mitoTEMPO, partially reversed dGTE2-induced decrease in ΔΨm (Figure 3c) and increase in mtROS (Figure 3d) but did not completely attenuate either mitochondrial measurement. Finally, we sought to understand whether the effect of dGTE on mitochondrial oxidative potential regulates HepG2 viability and apoptosis. In vehicle- and dGTE1-treated cells, MitoTEMPO pretreatment had no impact on HepG2 cell viability or apoptosis but significantly blunted dGTE2-induced cell viability and apoptosis (Figure 3e and 3f). Taken together, these findings indicate that dGTE1 and dGTE2 differentially impact HepG2 cell viability through regulating mitochondrial membrane potential and mitochondrial oxidative stress.

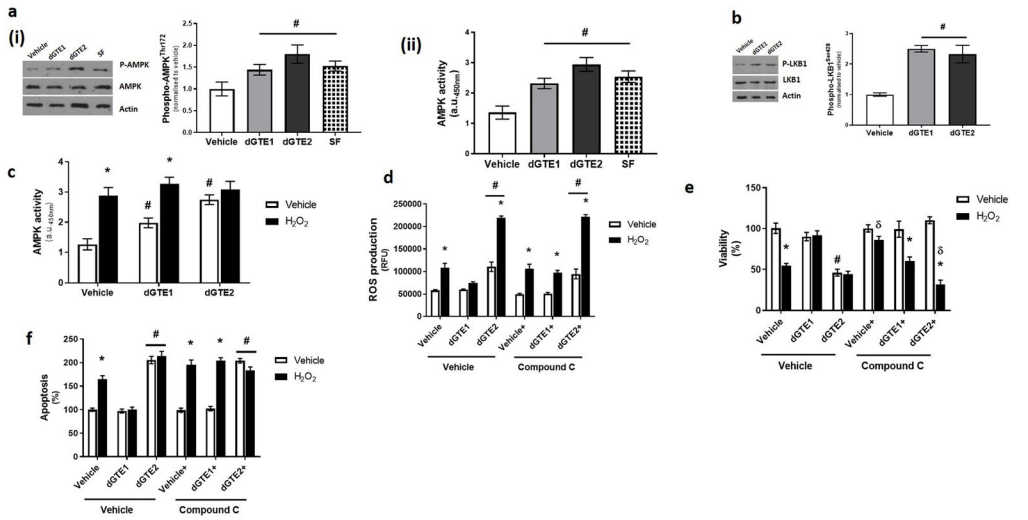

Figure 4. GTE2, but not GTE1, negatively regulates HepG2 cells through AMPK signaling. Panel *a-b:* HepG2 cells were exposed to dGTE1 and dGTE2 (1 μg·ml^{-1}), vehicle (H_2O), or serum free (SF) media as a positive control, for 24 h. Cells were then either harvested and assessed by immunoblot analysis for phosphorylated AMPK (Thr^{172}) and LKB1 (Ser^{428}) (a(i) and b) or assayed for AMPK activity using the CycLex kit assay and measuring tetra-methylbenzidine absorbance (a(ii)). A representative immunoblot is shown. *Panel c:* HepG2 cells were exposed to dGTE1 and dGTE2 (1 μg·ml^{-1}), or vehicle (H_2O), in the presence (closed bar) and absence (open bar) of H_2O_2 (0.8 mM) for 24 h. AMPK activity was assessed as *panel a(ii)*. *Panel d-f:* HepG2 cells were pretreated with the AMPK inhibitor, compound C (7.5 μM) for 30 min followed by exposure to dGTE1 and dGTE2 (1 μg·ml^{-1}), or vehicle (H_2O), in the presence (closed bar) and absence (open bar) of H_2O_2 (0.8 mM) for 24 h. ROS production (d), HepG2 cell viability (e) and apoptosis (f) were assessed using DCFDA, CCK8 and annexin V respectively. Data are presented as mean ± SEM. $^{*}p < 0.05$ versus vehicle for H_2O_2, $^{\#}p < 0.05$ versus vehicle for GTE, $^{\delta}p < 0.05$ versus vehicle for compound C.

dGTE2, but not dGTE1, negatively regulates HepG2 cells through AMPK signaling

Several studies demonstrate that activity of AMPK, a sensor of cell energy homeostasis and metabolism, is intrinsically linked with mitochondrial dysfunction, oxidative stress and cell apoptosis (Jeong et al. 2017; Alnahdi et al. 2019; Wang et al. 2019). Therefore, we next assessed the impact of dGTE1 and dGTE2 on AMPK activity by measuring phosphorylation (phospho-AMPKα (Thr^{172})) and activity of the serine/threonine kinase. Interestingly, both dGTE1 and dGTE2 increased AMPK phosphorylation (Figure 4a (i)) and activity (Figure 4a (ii)) to a level similar to the positive control (serum free media, SF). As anticipated, both dGTE treatments also increased phosphorylation of LKB1, a well-established activator of AMPK (Figure 4b) (Ducommun et al. 2014; Behunin et al. 2015). Interestingly, exposure to hydrogen peroxide significantly elevated AMPK activity in HepG2 cells, however this was attenuated by dGTE1 and unaffected by dGTE2 (Figure 4c). To investigate the impact of AMPK on dGTE-induced changes in cellular ROS, viability and apoptosis, cells were pretreated with the AMPK inhibitor (compound C) (Jeong et al. 2017) in the presence and absence of H_2O_2. In the absence of H_2O_2, AMPK inhibition had no impact on baseline levels of ROS (Figure 4d), HepG2 cell viability (Figure 4e) or apoptosis (Figure 4f). In the presence of H_2O_2, the AMPK inhibitor did not affect vehicle- or dGTE2-treated cells, but did prevent dGTE1 from protecting

Table 3. Bioavailability of ECGC in participants following oral supplementation of green tea extract versus control supplement. Healthy volunteers were given a dGTE1 or control supplement to consume and emergence of EGCG in plasma was measured over a 6 h period using LC/MS/MS. Control is a lipid coated (non-absorbable coating) dGTE1. Data are presented as mean ± SEM. $^{*}p < 0.05$ versus 0 h (pre-supplement).

	ECGC ($\mu g \cdot ml^{-1}$)	
Timepoint after consumption of supplement	Control	dGTE1
0 h	0.0017 ± 0.0000	0.0031 ± 0.0007
2 h	0.0094 ± 0.0038	0.1605 ± 0.0793
4 h	0.0555 ± 0.0095	0.2528 ± 0.0776*
6 h	0.0457 ± 0.0040	0.2631 ± 0.0605*

against H_2O_2-induced ROS accumulation (Figure 4d), reduced cell viability (Figure 4e) or increased apoptosis (Figure 4f). These studies therefore show that, although both dGTE1 and dGTE2 increase AMPK activity, the negative impact of dGTE2 on HepG2 cells is AMPK-independent, whereas the positive effect of dGTE1, in settings of oxidative stress, is AMPK-dependent.

Bioavailability of EGCG following consumption of dGTE1

Whilst these studies indicate the effect of dGTE1 and dGTE2 on hepatocytes, the liver would not necessarily encounter these extracts but would likely be exposed to the major components of dGTE including catechins. We therefore next sought to understand whether EGCG, a key component in green tea extract, was responsible for the protective effects observed following dGTE1 exposure (Mi et al. 2017; Zhao et al. 2017). Given the cytotoxic effects noted, it was deemed not ethical to assess the dGTE2 supplement in this study. A bioavailability study was first carried out, using dGTE1 in healthy participants, to study the emergence of the exogenous measurable compound within green tea extract, EGCG, in plasma at 2 h intervals after the consumption of the supplement at recommended doses. Bioavailability of EGCG was measured up to 6 h post-consumption in line with published studies showing that EGCG levels return to baseline by 8 h (Naumovski et al. 2015). At 2 h, there was an increase in the concentration of EGCG in the plasma which continued to rise at 4 and 6 h post-consumption of the supplement, to reach significance (Table 3). The maximal plasma concentration at 6 h following oral supplementation was 0.2631 $\mu g \cdot ml^{-1}$. These data indicate the EGCG concentration in the plasma from consumption of dGTE1 at this dosage was equivalent is 0.263 $\mu g \cdot ml^{-1}$ and that hepatocytes would indeed encounter the catechin EGCG, at this concentration.

EGCG protects against hydrogen peroxide-induced oxidative stress and cell death in a manner similar to dGTE1

Next, we therefore assessed the effect of EGCG, at the physiologically-relevant concentration of 0.263 $\mu g \cdot ml^{-1}$, on hepatic cell functions to understand whether this was responsible for the dGTE1-induced protection. HepG2 cells were exposed to EGCG for 24 h, to mirror dGTE1 studies, and cell viability and apoptosis, cellular and mitochondrial ROS, and AMPK signaling was assessed. Treatment with EGCG had no impact on baseline cellular (Figure 5a) or mitochondrial (Figure 5b) ROS accumulation,

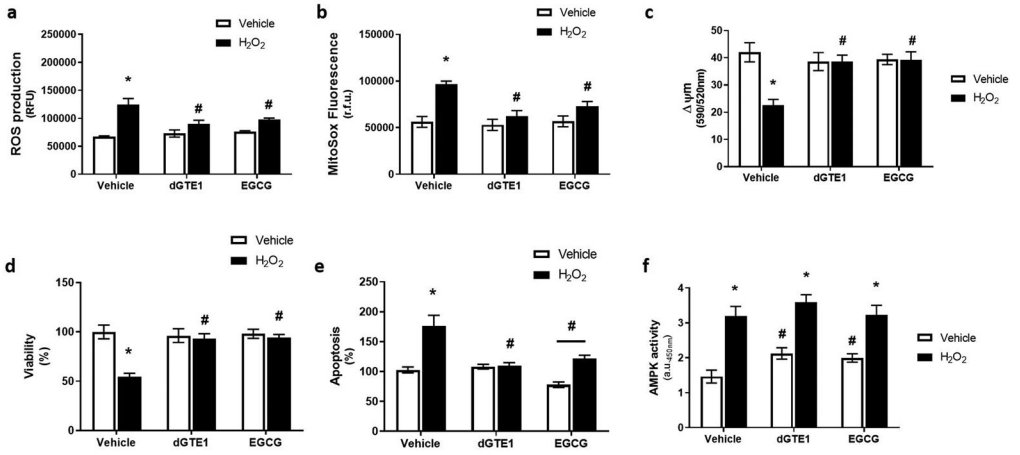

Figure 5. EGCG protects against hydrogen peroxide-induced oxidative stress and cell death in a manner similar to dGTE1. HepG2 cells were exposed to dGTE1 (1 μg·ml^{-1}) and EGCG (0.263 μg·ml^{-1}), or vehicle (H_2O), in the presence (closed bar) and absence (open bar) of H_2O_2 (0.8 mM) for 24 h. *Panels a and b:* Cellular and mitochondrial ROS were assessed using DCFDA and MitoSOX. *Panel c:* Mitochondrial membrane potential was assessed using JC-1. *Panel d-e:* HepG2 cell viability (d) and apoptosis (e) were assessed using CCK8 and annexin V respectively. Panel f: AMPK activity was assessed using CycLex kit assay. Data are presented as mean ± SEM. *$p < 0.05$ versus vehicle for H_2O_2, #$p < 0.05$ versus vehicle for GTE.

mitochondrial membrane depolarization (Figure 5c), viability (Figure 5d) or apoptosis (Figure 5e). Interestingly, EGCG exposure protected against H_2O_2-induced oxidative stress (Figure 5a and b) and mitochondrial membrane disruption (Figure 5c), as well as preventing cell death (Figure 5d) and apoptosis (Figure 5e). The catechin did, however, increase AMPK levels at baseline and was not able to impact H_2O_2-induced increase in AMPK activity (Figure 5f). These findings demonstrate that EGCG exposure, at physiologically-relevant, bioavailable concentrations, closely mirrors the protective effect of dGTE1 however it is acting through an AMPK-independent pathway.

dGTE2 contains stearic acid and higher levels of palmitic acid and caffeine than dGTE1

Our final set of experiments were performed using GC/MS to give qualitative identification and differences of the chemical composition of dGTE1 and dGTE2. A comparison of dGTE1 and dGTE2 demonstrated three specific differences observed at various retention times (Figure 6a, Table 4). A peak at retention time of 7.5 and 13 mins was present in both dGTE1 and dGTE2 and is therefore not discussed here. A higher level of caffeine and palmitic acid was observed in dGTE2 compared to dGTE1 at a retention time of 11.28 mins (Figure 6a and mass spectrum in Figure 6b) and 12.33 mins (Figure 6a and mass spectrum in Figure 6c) respectively. In addition, steric acid was identified in dGTE2 but not dGTE1 at a retention time of 13.7 mins (Figure 6a and mass spectrum in Figure 6d). This qualitative analysis indicates key differences in the chemical composition of dGTE1 and dGTE2 which are likely to be linked to the differing biological effects of each on HepG2 cells.

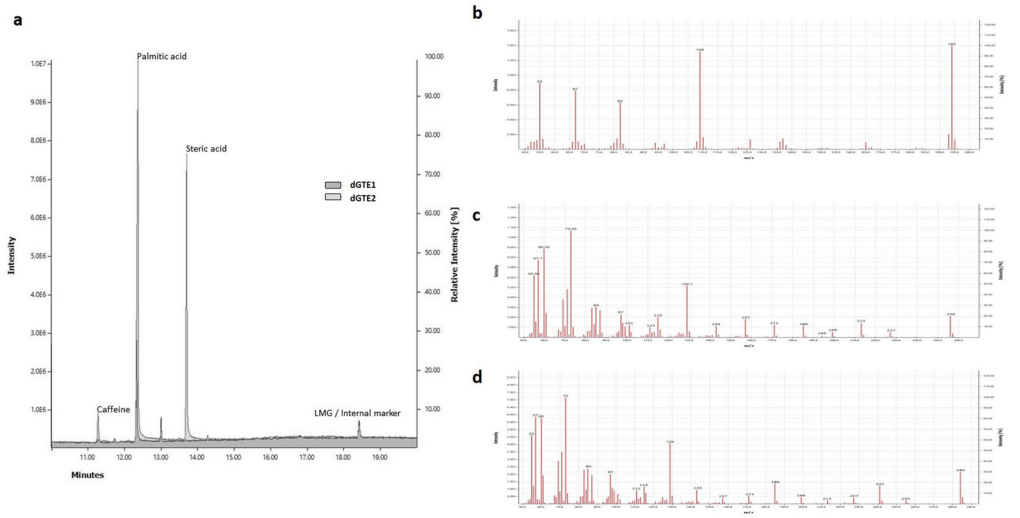

Figure 6. dGTE2 contains stearic acid and higher levels of palmitic acid and caffeine than dGTE1. Results obtained from Shimadzu QP2010 using ZB-1 column with method details given in Table 1. *Panel a:* Select region of typical total ion chromatogram to show chemical composition differences identified between dGTE1 and dGTE2. *Panel b:* Mass spectra for caffeine (major ions m/z 194, 126, 109) obtained from peak in panel a at retention time 11.28 mins. *Panel c:* Mass spectra for palmitic acid (major ions m/z 256, 213, 129) obtained from peak in panel a at retention time 12.33 mins. *Panel d:* Mass spectra for steric acid (major ions m/z 284, 241, 185) obtained from peak in panel a at retention time 13.7 mins.

Table 4. List of identified compounds *via* GC/MS analysis of green tea extracts (dGTE1 and dGTE2). The identification qualified by Match% NIST11 indicated NIST11 library spectrum similarity.

RT (mins)	Library/ID	Major Ions (m/z)	CAS	Match % NIST 11
11.28	Caffeine	194, 125, 109	58-08-2	89.10
12.33	Palmitic Acid	256, 213, 129	57-10-3	77.08
13.70	Stearic Acid	284, 241, 185	57-11-4	86.20
18.42	Internal Marker	330, 253, 165	129-73-7	82.92

Discussion

The present study sought to establish the effect of two dGTE on hepatocyte function using an *in vitro* model. We identified that the two extracts had opposing effects on HepG2 cell viability through apoptosis, cellular and mitochondrial oxidative stress and mitochondrial membrane depolarization. dGTE1 was protective against hydrogen peroxide-induced apoptosis and cell death, accumulation of ROS, and mitochondrial disruption, indicative of an antioxidant profile. In contrast, dGTE2 was cytotoxic with increased apoptosis and cell death, oxidative stress in the cell and within the mitochondria, and excessive mitochondrial membrane depolarization. Interestingly, both extracts increased activation of AMPK and upstream signaling, however the protective effect of dGTE1 was blocked by the AMPK inhibitor, compound C. Further study of the bioavailability of the key catechin in dGTE (EGCG) in humans indicated a physiologically-relevant concentration which was studied in the *in vitro* cell model. EGCG, at this concentration, exhibited a cytoprotective effect similar to that of dGTE1. These studies are the first to demonstrate opposing effects of two dGTE supplements, one which is

commercially-available and one which was purchased directly from the source, and establish the effect of dGTE on the liver at a cellular level.

Whilst these studies demonstrate a positive and negative effect of dGTE1 and dGTE2, respectively, on hepatocyte function, it is unlikely that the liver would be exposed to the extracts at these concentrations. GTE has been demonstrated to be comprised of a range of compounds, including catechins and polyphenols, although this varies greatly depending on the type of green tea studied and the processes used to prepare the extract (Green 2012). These compounds have been indicated to undergo sulfation, glucuronidation, and potentially o-methylation in the small intestine from where they are transported to the liver, *via* hepatic portal vein, for potential further metabolism (Meng et al. 2001). Furthermore, the intestinal microflora produces catabolites including phenylvaleric acid and phenylvalerolactones (Li et al. 2000). Together, this gives rise to at least 10 potential metabolic products which have been identified in plasma and urine following consumption of different products containing green tea extract (Stalmach et al. 2009). Furthermore, HepG2 cells express low levels of cytochrome P450 resulting in reduced metabolic capacity when compared to primary human hepatocytes (Westerink and Schoonen 2007). Therefore, to further understand the impact of dGTE1 and dGTE2 on HepG2 cells, it would be necessary to identify the compounds which comprise each extract, pinpoint the associated metabolites for each dGTE following consumption in the diet, and subsequently expose hepatocytes to these metabolites. Whilst this would give an understanding of the physiological effect of extracts, our small-scale bioavailability study establishes the concentration of EGCG in the plasma following consumption of dGTE1. We further demonstrate that exposure of hepatocytes to these EGCG concentrations is protective against hydrogen peroxide-induced apoptosis and cell death, accumulation of ROS, and mitochondrial disruption. The outcomes of this study are therefore closely linked to physiological effects seen following consumption of GTE in the diet, however further studies are needed to indicate the exact dGTE1 and dGTE2 metabolites which are responsible for the hepatocyte protective and damaging roles respectively.

Given the opposing effects of dGTE1 and dGTE2 on hepatocyte function, consideration is needed as to the mechanism for this difference. GTE has been studied extensively with regards to the composition and polyphenol chemistry. The beneficial properties of GTE are attributed to the abundance of polyphenolic compounds and specifically catechins, including (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin-3-*O*-gallate and the most abundant, EGCG (Green 2012). EGCG has been demonstrated to downregulate several molecular targets in pattern recognition receptor signaling pathways, such as TLR, IKKβ, IRAK-1 and TBK-1, associated with decreased inflammation (Pan et al. 2000; Youn et al. 2006; Kakuta et al. 2011). In the present study, the two dGTEs assessed contain a high % of EGCG with 571 mg dGTE1 delivering 70% or 400 mg EGCG versus 725 mg dGTE2, delivering 45% or 326.25 mg of EGCG. However, dGTE1 exerted a cytoprotective effect whilst dGTE2 was cytotoxic to hepatocytes. Interestingly, our findings demonstrate that the plasma concentration of EGCG, following dGTE1 consumption, is 0.2631 $\mu g \cdot ml^{-1}$ and that this concentration is sufficient to exert a cytoprotective effect on HepG2 cells in a similar manner to dGTE1 exposure. This bioavailability study was deemed not ethical for studying dGTE2, given the

cytotoxic effects identified in the *in vitro* studies. It is therefore not possible to confirm the bioavailability of EGCG following dGTE2 consumption. A review by United State Pharmacopeia established that GTE intake ranges widely depending on the supplement resulting in EGCG daily doses varying between 250 mg and 1800 mg (Oketch-Rabah et al. 2020). Interestingly, toxico- and pharmaco-kinetic studies demonstrate that catechin doses are a key determinant for the occurrence and severity of hepatotoxicity with a recommended safe adult intake level of EGCG at 338 $mg{\cdot}d^{-1}$ and an observed safe level of 704 $mg{\cdot}d^{-1}$ (Hu et al. 2018). Consideration is therefore needed with regards to the bioavailable EGCG levels following consumption of different dGTE compounds, and whether this can impact opposing effects on hepatocytes. However, irrespective of the mechanism through which the two different dGTE impact hepatic function, there is a need to consider the potential for hepatotoxicity in supplementation labeling. Indeed, our findings support the suggested labeling changes by the United States Pharmacopeia and the EFSA which state that the public should not consume dGTE if they have an existing liver condition, and should cease consumption of the supplement if they display symptoms of liver dysfunction (Oketch-Rabah et al. 2020; EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) et al., 2018).There are a range of signaling mechanisms through which GTE could impact hepatocyte function. RNA-seq transcriptomic studies in liver tissues of obese adult zebrafish implicate that GTE increases expression of genes associated with WNT/β-catenin and AMPK signaling (Zang et al. 2019). The latter was confirmed in C57BL/6 mice, where GTE supplementation upregulated AMPK and the upstream regulator, LKB1, in the liver (Santamarina et al. 2015; Bae et al. 2018). In hepatocytes, LKB1, activated by AdipoR2-mediated SIRT1 expression, migrates from the nucleus to the cytoplasm to phosphorylate the predominant AMPK subunit, AMPKα (Canto and Auwerx 2009; Shen et al. 2010; Lee et al. 2012). Once AMPKα is phosphorylated, the kinase is active and able to reduce lipogenic enzyme activity (ACC and FAS) and thus reduce the synthesis of fatty acid *de novo* lipogenesis (Kohjima et al. 2008). In our studies, both dGTE1 and dGTE2 significantly upregulated LKB1 and AMPK phosphorylation and activity, which indicates that the AMP activated kinase is not linked to the cytoprotective and cytotoxic effects of each extract. Interestingly, the AMPK inhibitor, blunted the protective effect of dGTE1, but had no impact on dGTE2-mediated toxic effects. Indeed, there are contradictory studies showing that AMPK activity is associated with apoptosis and oxidative stress in hepatocytes with some studies indicating a pro-apoptotic state (Jeong et al. 2017; Cui et al. 2018; Wang et al. 2019; Whang et al. 2019) and others suggestive of an antioxidant, cytoprotective effect of AMPK activation (Fan et al. 2018). In other cell types, AMPK activation is also linked with increased autophagy, mitochondrial dysfunction, ROS accumulation, apoptosis and cell death, potentially through elevated p53 and ROS production (Cui et al. 2018; Wang et al. 2019; Whang et al. 2019) however in primary cardiomyocytes, increased AMPK activity is linked with reduced disruption of mitochondria membrane potential and inhibition of mitochondrial oxidative stress levels (Lai et al. 2019). It is possible that AMPK acts as a 'sensor switch' and, depending on parallel signaling protein cascades such as mTOR, AKT and PI3K (Hawley et al. 2014; Zhang et al. 2016; Kazyken et al. 2019), the kinase can either positively or negatively affect cell status. In agreement with the diverse and complex outputs of the range of

AMPK-dependent downstream signaling profiles, our studies show that dGTE1 activates AMPK to exert a positive effect on hepatocyte function, such as oxidative status, mitochondrial function, and cell viability, whilst dGTE2-mediated AMPK activation is not associated with hepatocyte function. Furthermore, our findings demonstrate that dGTE1 exerts an AMPK-dependent protective effect on oxidative stress in HepG2 cells, whereas EGCG exposure alone protected oxidative stress in an AMPK-independent manner. It is therefore likely that EGCG is not the key antioxidant molecule present in dGTE1 and AMPK is not the only downstream signaling molecule activated in response to GTE supplementation. Other catechins present in GTE, at lower levels than EGCG, such as (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin-3-*O*-gallate could be present in differing concentrations in the two dGTE supplements studied (Green 2012). These molecules could act, in concert with EGCG, to attenuate oxidative stress in hepatocytes, however further study is needed to investigate the role of these less-considered catechins.

Interestingly, qualitative chemical analysis of each extract, using GC/MS, demonstrates the presence of steric acid, palmitic acid and caffeine in dGTE2, although the latter two are also found in dGTE1 but at low levels. This is in agreement with previous studies which show the presence of both steric acid and palmitic acid in leaf-grade variety green tea; steric acid is a commonly-used anti-caking and palmitic acid is the most common saturated fatty acid found in plants (Ahmad et al. 2015; Carta et al. 2017). *In vitro* studies by Garcia-Ruiz *et al* show that both palmitic acid and steric acid exposure can reduce the activity of OXPHOS complexes to lower cellular ATP levels and increase oxidative stress in HepG2 cells (Garcia-Ruiz et al. 2015). It is therefore possible that the presence of steric acid and increased levels of palmitic acid in dGTE2, compared to dGTE1, may cause fatty acid-induced cytotoxic effects observed in dGTE2-exposed HepG2 cells. Likewise, we demonstrate that the alkaloid caffeine is present in higher levels in dGTE2 than dGTE1. Previous studies indicate that caffeine ($\sim$8 $\mu g \cdot ml^{-1}$) can induce cytotoxicity in human hepatocarcinoma cells (Romualdo et al. 2020) whilst *in vivo* studies suggest that caffeine consumption can reduce oxidative stress and fibrosis in settings of liver disease such as cirrhosis (Amer et al. 2017; Arauz et al. 2017). Given this controversy regarding hepatic response to caffeine exposure, and that dGTE1 and dGTE2 are labeled as decaffeinated, it is unlikely that caffeine is responsible for the biological effects of GTE observed however further investigation is needed to establish the levels of caffeine in each extract. Indeed, analytical chemistry techniques have previously shown that decaffeinated tea still contains caffeine but at levels lower than 12 mg per serving, in contrast to up to 61 mg per serving in caffeinated teas (Chin et al. 2008). The opposing effects of dGTE1 and dGTE2 may be due to varying levels of other catechins in GTE (including (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin-3-*O*-gallate) as well as other polyphenols found in tea such as flavanols and their glycosides, and theogallin (Green 2012). Further analysis of the levels of these compounds in dGTE1 and dGTE2, as well as the bioavailability of each, would be needed to further understand the mechanism through which dGTE1 acts as a cytoprotective agent to abolish oxidative stress and mitochondrial dysfunction induced by hydrogen peroxide. Given the two dGTE supplements were sourced from different companies in different countries, it is also possible that dGTE2 contains various cytotoxic agents which are

accumulated throughout the extract processing procedure, whilst dGTE1 are not. Studies show that heavy metals, such as zinc, can be present in tea plants where plantations are in close proximity to heavily polluted areas or landfill sites (Rezaee et al. 2014). Furthermore, many teas such as Rooibos tea have been shown to have a high microbial load which is, in part, linked to the tea fermentation, pasteurization and drying processes (Gouws et al. 2014). In other teas, a range of fungi which contain aflatoxins, mycotoxins such as fumonisins, and toxigenic fungi molds such as *A. Flavus*, *A. parasiticus*, and *Penicillium* spp (Trucksess and Scott 2008). Fumonisins and aflatoxins have been demonstrated to inhibit cell proliferation (Cetin and Bullerman 2005; Chen et al. 2016) and increase apoptotic cell death in primary and cultured hepatocytes including HepG2 cells (Kang et al. 2016; Wentzel et al. 2017; Meneely et al. 2018). The mechanism for this is unclear however it is possible that these mycotoxins are present in dGTE2, but not dGTE1, and therefore could be responsible for the opposing cytotoxic effects of the extracts.

In conclusion, our findings demonstrate that a commercially-available dGTE supplement causes mitochondrial disruption, oxidative stress and cell death in a human cell model, whilst a dGTE supplement used from source reduces hepatocyte damage in settings of oxidative stress. These studies highlight a key difference between two green tea supplements and has potential implications for the manufacturing and processing industry associated with supplementation.

Acknowledgements

We would like to acknowledge Jack Weekes who carried out the pilot bioavailability study.

Declaration of interest

Biocare Ltd was one of the sources of green tea extract and funded part of the research project however the company had no input into the study conceptualization, methodology approach, data analysis, manuscript writing or editing.

Funding

This material is based on work supported by Diabetes UK Grant 15/0005284 (H.Chichger) and Biocare Ltd. (J.Roberts).

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Justin Roberts: Conceptualization, data curation, funding acquisition; writing – review and editing.

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