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A randomized, placebo-controlled, double-blind study on the effects of (−)-epicatechin on the triglyceride/HDLc ratio and cardiometabolic profile of subjects with hypertriglyceridemia: Unique in vitro effects

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ABSTRACT

Background: Cardiometabolic disruptions such as insulin resistance, obesity, high blood pressure, hyperglycemia, and dyslipidemias, are known to increase the risk for cardiovascular and metabolic diseases such as type 2 diabetes mellitus and atherosclerosis. Several screening tools for assessing cardiometabolic risk have been developed including the TG/HDLc ratio, which has been, demonstrated to possess a strong association with insulin resistance and coronary disease.
Dietary modifications, together with regular moderate exercise have proven to be effective in attenuating cardiometabolic disruptions. However, they often exhibit poor long-term patient compliance. Nutraceutics, including (−)-epicatechin (EPI), have gained increasing interest as coadjuvant effective and safe therapies that are able to attenuate hypertension, hyperglycemia, hyperinsulinemia, hypertriglyceridemia and hypoalphalipoproteinemia.
Methods: The aims of this study were: 1) to compare the in vitro effect of EPI vs. (+)-catechin on fructose induced triglyceride accumulation and mitochondrial function in Hep2 cells in culture, 2) to evaluate the efficacy of EPI treatment in reducing fasting blood triglycerides and improving the TG/HDLc ratio in hypertriglyceridemic patients with a total daily dose of 100 mg of EPI. Secondary clinical variables included total cholesterol, LDLc, fructosamine, glucose, insulin, and high sensitivity C-reactive protein blood levels.
Results and conclusion: Our results provide preliminary evidence as to favorable effects of EPI on glycemia homeostasis, lipid profile and systemic inflammation such bioactive actions are not *class-effects* (i.e. limited to their antioxidant potential) but instead, may result from the specific activation of associated downstream signaling pathways since catechin has no effects.

1. Introduction

Metabolic syndrome (MS) comprises a cluster of cardiovascular and metabolic alterations such as insulin resistance, obesity, high blood pressure, altered glucose homeostasis and atherogenic dyslipidemias, among others that increase the risk for cardiovascular and metabolic diseases such as type 2 diabetes mellitus (T2DM) [1]. Although many of the underlying mechanisms of the MS and its comorbidities have yet to be fully established and are likely complex, insulin resistance and the resulting hyperinsulinemia have been proposed as key pathogenic factors [2]. As a result, screening tools for assessing cardiometabolic (CM) risk often focus on fasting glycemia and components of the lipid profile such as triglycerides (TG). Within the latter, recent studies have proposed the use of the TG/HDLc ratio (rather than serum TG or high-density lipoprotein cholesterol –HDLc-alone) as a strong and independent surrogate marker of CM diseases since this index is associated with both insulin resistance and coronary disease [3–8].

At the early stage of the MS, lifestyle adjustments [9] including the habitual practice of moderate to intense physical exercise, together with dietary modifications, have proven to be effective in reducing its progression [10–13]. In fact, in the Diabetes Prevention Program [14], diet and exercise were more effective than metformin in preventing the development of T2DM. However, such therapeutic lifestyle changes

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are frequently difficult to sustain due to poor long-term patient compliance. On the other hand, pharmacological interventions targeting weight reduction (the cornerstone of the MS treatment) have been proven to either exert limited benefit or be associated with significant secondary and/or adverse effects [15]. Thus, novel therapeutic strategies that safely and effectively limit the development of MS are needed. In this regard, natural functional foods or specific bioactive components of such [16], i.e., nutraceutics, have gained interest.

Complex mixtures of polyphenols extracted from natural products such as cacao or green teas have been observed to exert beneficial metabolic effects. Cacao-derived extracts have been proven to exert significant benefits on MS components such as attenuating high blood pressure, hyperglycemia, hyperinsulinemia, hypercholesterolemia, and hypertriglyceridemia, and increasing HDLc levels [17–19]. Similar claims have been made for green tea concentrates [20] however, polyphenol mixtures are not standardized. It would be preferable to identify a single flavonoid as a potential therapeutic compound to be used for MS. However, there is almost no data on whether there are variances in metabolic activities of the specific flavonoid components of available polyphenol extracts. While green tea extracts in general and one component therein, epigallocatechin gallate in particular, have been shown to exert therapeutic effects in animal models of MS, clinical testing of both green tea extracts and EGCG in humans with MS have been inconclusive, demonstrating no consistent effect on MS biomarkers [21]. With respect to specific flavonoids found in cacao, (−)-epicatechin (EPI) has demonstrated in preclinical studies [17,18] and in a pilot study in humans [24] to improve glycemia, HDLc, and TG. However, the potential of pure EPI to beneficially impact components of MS in humans and biomarkers such as the TG/HDLc has yet to be explored.

We have previously demonstrated that EPI effects are likely induced by the activation of a cell membrane receptor and its subsequent associated signaling pathways, thus the resulting health benefits do not reflect mere antioxidant effects but rather indirect actions through the modulation of regulatory systems involved in mitochondrial biogenesis and function [23,25], including antioxidant systems such as superoxide dismutase [26,27]. To answer such questions and pre-evaluate the potential of specific flavanols to ameliorate TG levels in human subjects, cell culture systems can be used in particular, the human hepatic cell line HepG2.

Given the aforementioned background, the primary objectives of this study were: 1) to evaluate in HepG2 cells the capacity of EPI and (+)-catechin (CAT) to modulate fructose-induced increases in TG content and levels of mitochondrial oxidative stress and, 2) to evaluate the efficacy of a 4-week EPI treatment in reducing fasting plasma TG in human subjects with hypertriglyceridemia and to assess changes in the TG/HDLc ratio. Secondary clinical endpoints included the evaluation of EPI effects on total cholesterol, LDLc as well as fructosamine, glycemia and insulin (as indicators of glucose metabolism profile status) and high sensitivity C-reactive protein (hsCRP) levels as indicator of changes in inflammation and cardiovascular risk.

2. Methods

2.1. Cell culture

HepG2 cells were obtained from the American Type Culture Collection (ATCC). Cells were grown to 75% confluency in Dulbecco's modified Eagles media supplemented with 10% fetal bovine serum and exposed to fructose (5 mM) to induce increases in TG levels alone or in combination with EPI (10 μM) or CAT (10 μM) for 72 h. Medium was replaced with fresh fructose and flavanols twice daily.

2.2. TG content

For TG determination, cells were homogenized and fat was solubilized in 200 μL of solution containing 5% Nonidet P-40 in water. Samples were slowly exposed to an 80–100 °C water bath for 2–5 min until the Nonidet P-40 became cloudy and then cooled down to room temperature. Samples were centrifuged for 2 min to remove insoluble material. TG determination was performed using VetAce autoanalyzer in the initial breakdown into fatty acids and glycerol [28]. Glycerol was oxidized to generate a product that reacts with the probe to generate color at 570 nm.

2.3. Mitochondrial oxidative stress

Hep2G mitochondrial oxidative stress levels were determined using specific mitochondria-targeted fluorescent dye (MitoSOX®, Thermo Fisher Scientific, Inc.) as per the manufacturer's instructions. Briefly, HepG2 cell cultures were washed with warm phosphate balanced solution, incubated with 5 μM of the cell-permeant fluorescent probe for 30 min at 37 °C, protected from light. After incubation, cells were washed with warm buffer and total fluorescence determined employing a microplate reader. Fluorescence levels were expressed as total fluorescence intensity normalized to milligram of soluble protein.

2.4. Human studies

A randomized, multicenter, placebo-controlled, double-blind trial was implemented in subjects with hypertriglyceridemia (see Consort flow diagram, Fig. 1). Volunteers were screened at two clinical sites: the Pranav Diabetes Centre and the Cosmopolitan Medical Centre (both located at Bangalore, India). Subjects with hypertriglyceridemia (serum levels between 200 and 499 mg/dL) who were not currently on pharmacologic treatment for dyslipidemia (with the exception of statins at a stable dose ≥6 weeks prior to screening), and aged 18–55 years, were enrolled, irrespective of their gender, after signing a written informed consent. Non-inclusion criteria included participation in another clinical trial in the previous 30 days, high cardiovascular risk, arterial hypertension (≥140 systolic and/or ≥90 diastolic mm Hg), history of stroke, transient ischemic attack, unstable cardiac disease, abnormal EKG, coagulopathy or anticoagulant medication, pancreatitis, uncontrolled diabetes (HbA1c >9% and/or fasting glycaemia >200 mg/dL) or current insulin therapy, hypoglycemia, renal failure (GRF <60 mL/min), known HIV or hepatitis B or C infection, and consumption of ≥14 alcohol drinks per week, the use of insulin, daily aspirin, coumadin, or any other anti-platelet or anti-clotting therapy as well as atypical antipsychotics, beta-blockers, glucocorticoids, isotretinoin, and tamoxifene.

2.5. Sample size calculation

A total sample size of 30 patients was estimated to provide 80% power and 95% reliability (considering a 1:2 placebo:treatment ratio) with a mean of 32% reduction in serum TG. Such decrease was observed in a prior pilot study consisting in daily consumption of oral EPI for seven days (Gutiérrez-Salmeán G, unpublished data).

2.6. Cardiometabolic assessment

Fasting blood samples were collected by venipucture. Plasma was obtained after centrifugation at 3500 rpm for 15 min. TG, total cholesterol (TC), HDLc, glucose, fructosamine were determined using enzymatic colorimetric kits. LDLc was measured using gradient gel electrophoresis. Insulin and hsCRP concentrations were determined by ELISA kits. Non-HDLc was estimated by subtracting HDL from TC. Systolic and diastolic blood pressures were measured by conventional sphygmomanometry. Heart rate and body temperature were also recorded.

2.7. Treatment

EPI and placebo capsules were prepared in a Good Manufacturing Practices (GMP) facility. First, EPI was dissolved in ethanol and then it was treated with charcoal and filtered to remove insoluble materials. The solvent was exchanged with purified water and dried by lyophilization. The re-purified compound was tested in a GMP certified analytical lab using HPLC methodology. Specifications required >90% purity, <5% of the enantiomer and 5% CAT. It was also tested for other characteristics typical in GMP materials (identification by 1H nuclear magnetic resonance spectroscopy, infrared; water content by Karl Fischer titration; ethanol content by gas chromatography; and the general USP tests of residue on ignition and heavy metals). Based on test results, a certificate of analysis was generated and a percent content by weight was calculated. Microbiological tests for aerobic microorganisms, yeast and molds were negative (<10 cfu/g). Tests for *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* species and *Staphylococcus aureus* were also negative. Cardero Therapeutics Inc. (Los Altos Hills, CA) conducted the purification and formulation of EPI.

Condition assignments were performed with a table of random numbers generated by statistician at I5 Clinical Research Pvt. Ltd., patients were assigned randomly, in a 2:1 ratio of Treatment: Placebo. All investigators and subjects remained blind to condition assignment per protocol. EPI was supplied in hard gelatin capsules as well as the inactive placebo. To maintain a double-blind intervention all capsules were white opaque cap and white opaque body. After baseline measurements, subjects were randomized into two arms, 20 subjects for EPI and 10 for placebo. Subjects were instructed to take for a total of 4 weeks, two capsules of 25 mg EPI or placebo, twice a day orally, 30 min before meals (lunch and dinner) thus, yielding the total daily dose of 100 mg of EPI. All subjects were instructed to ingest a "healthy" diet limiting if possible, the ingestion of known sources of flavanols such as berries and green tea.