N-oleoyl-phosphatidyl-ethanolamine and epigallo catechin-3-gallate mitigate oxidative stress in overweight and class I obese people on a low-calorie diet.

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ABSTRACT

Oxidative stress and lipid peroxidation are considered key factors linking obesity with its associated complications. Epigallo catechin-3-gallate (EGCG) and oleoylethanolamide (OEA), together with its phospholipid precursor N-oleoyl-phosphatidylethanolamine (NOPE), are nutritional compounds that might improve the oxidative stress status of obese people. Unfortunately, the bioavailability of these compounds is low; however, the co-administration of NOPE with EGCG has been shown to ameliorates both the plasma availability of EGCG and the intestinal levels of NOPE in rats.

This double-blind, placebo-controlled study investigated the effects of 2 months' supplementation with EGCG complexed with NOPE, combined with moderate energy restriction, on plasma oxidative status of overweight and class I obese subjects. 138 subjects (BMI: 25 - 35 kg/m^2) were recruited and randomized into two groups: the first (n = 67) received caps of placebo and the second (n = 71) caps of an oily dispersion of EGCG complexed with NOPE for two months. Subjects' supplementation was combined with moderate energy restriction (-800 kcal/d).

Plasma oxidative status was determined by measuring the levels of oxidized LDL (Ox-LDL), malondialdehyde and reactive oxygen metabolites, and by calculating the lag-time and the slope of Cu-induced lipid peroxidation kinetics. 116 subjects (27 M/89 F) completed the supplementation period, 49 in the placebo group and 67 in the treated group. Treatment induced a similar significant weight reduction in the two groups. Moreover, we found the mean changes of Ox-LDL significantly lower and the mean changes of antioxidant capacity (lag-time) significantly higher in NOPE-EGCG group than in placebo group (treatment effect mean difference : -3.15 UL, $p<0.044$ and +5.37 min, $p<0.0347$; respectively). EGCG plasma levels were detectable only after two months of NOPE-EGCG-diet. The NOPE-EGCG integration to a low energy diet seems therefore useful for ameliorating oxidative stress related markers, which are concomitant causes of obesity-induced disorders.

Key words: lipid peroxidation, oxidative stress, obesity, N-oleyl-phosphatidylethanolamine, oleoylethonolamide, epigallo catechin-gallate.

INTRODUCTION

Overweight and obesity are associated with an increased risk to develop cardiovascular diseases, the clinical manifestations of atherosclerosis. In addition, the accumulation of abdominal fat is a major contributor to the development of a series of metabolic disorders recognized as risk factors for atherosclerosis, such as inflammation, hypertension, dyslipidemia, and insulin resistance. Several lines of evidence show that oxidative stress and lipid peroxidation may be unifying mechanisms in the development of obesity-related metabolic disorders [1,2]. Oxidative stress is an imbalance between tissue oxidants [free radicals, reactive oxygen species, (ROS) and reactive nitrogen species (RNS)] and antioxidants, which has been repeatedly demonstrated to be involved in atherogenesis [3] and several diseases [4]. In this condition, ROS promote lipid peroxidation resulting in chain reactions that generate a sustained increase in reactive compounds able to oxidize macromolecules such as lipids, proteins and DNA. Of the many biological targets of oxidative stress, lipids, especially polyunsaturated fatty acids (PUFA), are the most involved class of biomolecules. PUFA peroxidation gives rise to several secondary toxic products that amplify oxidative damage, such as lipid peroxides and aldehydes like malondialdehyde (MDA). At blood level, the peroxidation of lipoproteins, and in particular LDL fraction, induces a number of changes in their physical-chemical properties able to promote and exacerbate atherosclerosis [5].

Because overeating is one of the major causes of body fat increase and consequently of plasma oxidative stress, a diet restricted in energy intake and rich in antioxidant is the basis for the treatment of overweight and obesity. Researchers have been recently focusing on specific nutritional compounds capable of increasing plasma antioxidant defenses and/or inducing a reduction in food intake. Two of these compounds are epigallo catechin-3-gallate (EGCG) and oleoylethanolamide (OEA), together with its phospholipid precursor N-oleoyl-phosphatidylethanolamine (NOPE).

EGCG is the most abundant and strongest bioactive catechin of green tea. The potential health benefits ascribed to EGCG include antioxidant effects, enhancement of weight loss, improvement of insulin sensitivity and cardiovascular health, cancer chemoprevention, and others [6]. The role of EGCG in reducing lipid peroxidation, particularly LDL oxidation has been reported from several in vitro and animal, but limited clinical studies [7]. NOPE and OEA are present in plant and animal foods [8] and are produced and metabolized by humans [9,10]. NOPE is routinely metabolized "in vivo" by phospholipase D to phosphatidic acid and OEA [9]. Both phosphatidic acid [11] and OEA [12,13] possess antioxidant activity *in vitro*. Moreover, OEA can exert other beneficial effects on obese people, such as the modulation of lipid metabolism and energy intake [10,14]. Previous studies demonstrated that the bioavailability of both these compounds is low when administered alone as an oral supplement [15,16]; differently, the co-administration of NOPE with EGCG has been shown to ameliorates both the plasma availability of EGCG and the intestinal levels of NOPE in rats [16] through mechanisms that increase EGCG intestinal permeability and protect NOPE from the digestive enzyme-induced hydrolysis [17].

We previously evaluated [18] in a parallel-arm, double-blind, placebo-controlled design, the effects of 2-month administration of an oily NOPE-EGCG complex in healthy, overweight or class I obese people (106 females and 32 males). Results indicated that the assumption of a NOPE-EGCG complex improved compliance with diet, insulin resistance, depressive symptoms and severity of binge eating [18]. However, in this previous study we didn't investigate the effects of this complex on oxidative stress.

Therefore, the aim of this ancillary study was to evaluate in the same healthy overweight and class I obese subjects, the potential beneficial effects of 2 months' co-supplementation with EGCG and NOPE, combined with moderate energy restriction, on plasma oxidative status.

SUBJECTS AND METHODS

Subjects

Subject enrolment criteria has been previously described [18]. Briefly, eligible participants were recruited from the local population and underwent medical screening according to the following inclusion criteria: body mass index between 25 kg/m^2 and 35 kg/m^2 (i.e. overweight or class I obese people according with World Health Organization[19]) apparently health, not currently pregnant, normally menstruating, non-smokers, not taking medication, not being on a restricted diet, not drinking more than 1 cup of tea/day and not using dietary supplements during the three previous months. Patients were excluded from the study if they had a history or current diagnosis of major depressive disorder bulimia, panic disorder, obsessive compulsive disorder, post-traumatic stress disorder, bipolar I or II disorder, or schizophrenia. No psychoactive drugs, including anti-obesity agents, were permitted throughout the study. All subjects had to give complete medical histories and undergo physical examination, anthropometric assessment and routine laboratory tests. The study protocol was approved by the Medical Ethics Committee of the Policlinico San Matteo in Pavia and each subject signed a consent form stating the purpose of the study and the sampling required.

Study design

In this ancillary, double-blind and placebo-controlled study, subjects were randomly divided into 2 groups, as previously described [18]: NOPE-EGCG complex group (71 subjects, 18 men, 53 women, mean age 38 ± 10 years) and placebo group: (67 subjects, 14 men, 53 women, mean age 41 ± 11 years). Subjects received randomly one capsule of PhosphoLEAN™ orally twice daily — before lunch and dinner — or an identical placebo, for 2 months. The commercially available

supplement PhosphoLEAN™ was a soft-gel capsule containing 85 mg of NOPE and 14 mg of N-palmitoyl-phosphatidylethanolamine extracted from soy lecithin, and 121 mg of a dry green tea extract standardized at 50 mg of EGCG; the capsules were manufactured by GELFIPHARMA Lodi, Milan - Italy on behalf of CHEMI Cinisello Balsamo, Milan - Italy. Placebo contained 200 mg of soybean lecithin in soybean oil. Compliance was assessed by counting leftover capsules.

Subjects' supplementation was combined with a 2-months' period of moderate energy restriction (-800 kcal/d). Macronutrient content of hypo-caloric diet, expressed as percentage of ingested energy, was 25% fat, 60% carbohydrate, and 15% protein. At the end of the nutritional intervention, detailed food records were kept by the subjects on three day non-consecutive (two weekdays and one weekend days). The energy and nutrient contents of these records were estimated using Italian National Research Institute for Food and Nutrition database [20].

Body composition

Anthropometric measurements were taken between 9 and 11 a.m. after an overnight fast. Body weight (Kg) was measured on a standing balance and height (m) by using a standing upright scale. Body Mass Index (BMI) was calculated by dividing weight (Kg) by height squared (m^2). Waist circumference was measured at the midway point between the lowest rib and the iliac crest to the nearest 0.1 cm.

Blood collection and analyses

Overnight fast blood was drawn from subjects (12 hours without food) in the morning at study entry and after the 8 weeks of treatment. Moreover, subjects refrained from participating in any form of exercise for 48 h before the study and were tested during the early follicular phase of their menstrual cycles (days 3-10). Blood was collected into EDTA tubes for hematological

procedures. Blood samples were also collected into tubes containing heparin or no anticoagulant for the determination of plasma oxidative status; the samples were processed and immediately centrifuged twice (1000 x g for 15 min) with refrigeration, and aliquots of the plasma and serum were immediately frozen in liquid nitrogen and stored under argon at - 80°C until use.

Oxidized LDL (Ox-LDL) levels were determined by ELISA Kit accordingly with manufactures instruction (Mercodia, DBA, Italy). Plasma levels of malondialdehyde (MDA) and reactive oxygen metabolites (d-ROMs) were evaluated by means of commercially available colorimetric assay kits (OXIS International, Portland, OR, USA and Diacron International, Grosseto, Italy; respectively).

Copper-stimulated plasma lipid peroxidation was determined by fluorescent method [21–24]. Briefly, peroxidation kinetics were determined by estimating protein lipofuscin-like fluorophores formed by the reaction of aldehydic lipid oxidation products with protein (λ excitation = 360 nm; λ emission = 460 nm, T = 37 °C), every 15 min for 480 min. The length of the lag phase of inhibited lipid peroxidation was measured (lag-time), as well as the rate of lipid peroxidation during the propagation phase (slope). The lag-time value is an index of plasma total antioxidant capacity. Vitamin E (α-tocopherol) [25] and EGCG [31] were measured by reverse-phase high performance liquid chromatography methods. The ratio of vitamin E (μmol/L) to total neutral lipid (cholesterol + triglycerides, mmol/L) was also calculated.

All frozen samples were thawed only once, at the time of the assays, and, samples collected at baseline and at the end of the study from each subject were analyzed in duplicate in the same assay to eliminate inter-assay variability.

Statistics

Data are presented as mean and standard deviation (SD). Data were checked for normality and skewed parameters were mathematically transformed before statistical analysis. At baseline,

the differences between placebo and NOPE-EGCG groups were tested using Student's *t* test. Mean changes over time and 95% CI were calculated in each group, as final value minus baseline value. Mean differences between changes were computed with their 95%CI to quantify the treatment effect. The treatment effect was analyzed by the Student's *t* test. Spearman's correlation analysis was used to quantify the relationship between treatment-induced changes in the measured parameters. StatistiXL (version 1.9; StatistiXL, Western Australia) was used for computation. A 2-sided p-value<0.05 was considered statistically significant.

RESULTS

Table 1 showed demographic, anthropometric and biochemical characteristics of the NOPE-EGCG and placebo group, at baseline (T_0). Mean lipid panel parameters, glycemic and inflammatory status levels were substantially within the relevant reference interval or under/above cut-off value both in the NOPE-EGCG and in the placebo group and did not differ significantly. No significant differences in oxidative status parameters were found in both groups.

The mean prescribed caloric intake, at T_0, was 1475 $\pm$ 28.6 kcal/d and 1487 $\pm$ 24.2 kcal/d in the placebo and NOPE-EGCG group, respectively.

Out of 138 participants entering the study, 116 (27 M/89 F) completed the 8-week supplementation period, forty-nine (73%) patients in the placebo group and sixty-seven (94%) in the treated group. The mean energy intake after 8 weeks of treatment was 1990 $\pm$ 82.2 kcal/d and 1912 $\pm$ 65.4 kcal/d in the placebo and NOPE-EGCG groups, respectively (p = 0.459). As designed, macronutrient distribution was similar in both diets, and no statistical differences were found in the energy provided by carbohydrates, lipids and proteins (data not shown).

As previously reported, the treatment induced a significant weight reduction in both groups (-2.3% ± 3.5% and -3.7% ± 3.3% placebo and NOPE-EGCG groups, respectively); but the weight changes were not significantly different between the groups [18].

The ratio of vitamin E to total neutral lipid and MDA levels didn't vary significantly in both groups (data not shown). In table 2 are reported the mean changes (final value minus baseline value) of plasma peroxidation kinetic parameters (lag-time and slope), d-ROMs and Ox-LDL. The mean changes of these parameters were similar in the NOPE-EGCG group and in the placebo group, except for lag-time and Ox-LDL. The administration of the NOPE-EGCG complex significantly improved plasma antioxidant capacity (lag-time) and reduced Ox-LDL levels with respect to the hypocaloric diet alone.

EGCG plasma levels were not detectable after the placebo-diet treatment, while a substantial amount of this polyphenol is found in patient plasma treated with the NOPE-EGCG-diet (10.1 ± 3.4 nmol/L). The increase in EGCG was associated with changes in lag-time and oxidized LDL in the NOPE-EGCG group ($r_s = 0.48$; $p < 0.05$ and $r_s = -0.58$; $p < 0.01$, respectively).

DISCUSSION

In a previous study we demonstrated that 2-month administration of an oily NOPE-EGCG complex improved compliance with hypocaloric-diet, insulin resistance, depressive symptoms and severity of binge eating in healthy overweight and class I obese people [18]. The results of the ancillary present study indicate that the treatment with NOPE-EGCG complex ameliorates concomitantly plasma oxidative status too in these subjects.

It is well known that oxidative stress is a factor linking obesity with its associated complications [1] and that oxidative stress-induced oxidation of low-density lipoprotein (LDL) to Ox-LDL is one of the first steps of atherogenesis[26]. Nowadays, according to the European Food Safety Authority (EFSA), Ox- LDL is considered a reliable marker for oxidative damage *in vivo* [27]; however, over the last four decades, an extensive body of literature regarding oxidative stress-induced lipid peroxidation has shown its important role in the dysregulation of several biological processes [28]. For this reason, we measured also other oxidative stress markers to have supportive evidences. In particular, MDA [29] and d-ROMs [30–32], are widely used as markers of the extent of plasma lipid peroxidation, while the duration of latency phase (lag-time) of lipid peroxidation kinetics is considered an index of total plasma antioxidant capacity [21,22,33,34] . The supplementation with NOPE-EGCG had significantly decreased the Ox-LDL levels and increased antioxidant capacity with respect to hypocaloric diet alone.

Previous studies have shown that oxidative stress increases with increasing BMI [35] and that intentional weight loss mitigates oxidative stress in obese people [36] and reduces Ox-LDL levels in obese children and adolescents [37]. Nevertheless, as mentioned above, we found no differences in body weight changes between the NOPE-EGCG group and the placebo group [18]. The absence of effect on weight loss of the treatment with NOPE-EGCG has been confirmed by Mangine et al. who conducted a study that assessed the effects of 8-weeks supplementation with a daily intake of 120

mg of NOPE and 105 mg of EGCG in combination with a low caloric diet on body composition and dietary compliance in healthy, overweight adults [38].

The antioxidant activity of EGCG and other green tea polyphenols (GTPs) has been widely investigated both *in vitro* and *in vivo* [7]. EGCG has both direct and indirect antioxidant activities and its beneficial effects seem due to a combination of both mechanisms [39]. *In vitro* and animal studies have shown that EGCG is able to chelate redox-active metal ions, such as iron and copper, to scavenge oxygen and nitrogen free radicals and reactive oxygen species (ROS), and, as a consequence, to inhibit lipid, protein and DNA peroxidation, whereas it does not affect the propagation of peroxidation processes [40,41]. The indirect antioxidant activity of EGCG is related to inhibition of redox-sensitive transcription factors, inhibition of pro-oxidant enzymes, induction of Phase II enzymes and up-regulation of antioxidant enzymes [42–44] . All these EGCG activities may have contributed to the increased latency phase (lag-time) of plasma peroxidation kinetics in NOPE-EGCG group.

Furthermore, EGCG can bind to LDLs and this interaction reduces the susceptibility to oxidation of lipids and proteins of these particles [45]. It is well known that catechins and proteins have strong interactions through hydrogen bonding and/or hydrophobic attractions. In addition, gallated catechins interact also with the trimethylammonium group of phosphatidylcholine [46], which is the major phospholipid in human LDLs . Therefore, EGCG's interaction with LDL apolipoproteins and phosphatidylcholine, by promoting free radical neutralization and vitamin E regeneration from alpha- tocopherol radicals [47], might have contribute to the improvement of serum ox-LDL levels in NOPE-EGCG group.

Despite the large body of evidence for the antioxidant effect of EGCG *in vitro*, the results of human intervention studies are contradictory and seem to be dependent on the dose ingested, the diet, the measured markers and the extent of oxidative stress in the subjects. [48]. In particular, GTPs

and EGCG seems reduce lipid peroxidation processes [49], especially in subjects that exhibit oxidative stress-related risk factors. In addition, the level of plasma lipid peroxidation markers have been associated with those of circulating lipids [50,51]. In particular, it has been demonstrated that both MDA and d-ROMs levels [50] correlated positively and significantly with triglyceridemia, likely because triglycerides are the main form of storage and transport of polyunsaturated fatty acids. In this study we consider healthy, non-smokers, normo-triglyceridemic overweigh or class I obese subjects and, as for weigh, we found no differences in triglyceridemia changes between the NOPE-EGCG group and the placebo group [18]; therefore, both these factors could be contribute to the lack of effects of NOPE-EGCG on MDA and d-ROMs levels.

Furthermore, also the NOPE present in the NOPE-EGCG complexes could have contribute to the increase of lag-time. These complexes are composed by a mixture of N-acyl-phosphatidylethanolamines (NAPEs) constituted mostly by N-oleoyl-phosphatidylethanolamine (85%), and by N-palmitoyl-phosphatidylethanolamine (14%) and trace amounts of other NAPEs. NAPEs are hydrolyzed in the gut to phosphatidic acid and the corresponding acylethanolamides (AEAs); in our case OEA and in lesser extent palmitoylethanolamide (PEA). These AEAs are lipid mediators that regulate a plethora of physiological functions [52]. OEA and PEA are considered endocannabinoid-like lipids because of their structural analogy with the endocannabinoid arachidonoylethanolamide (also referred as anandamide) which is a full agonist of cannabinoid receptor type 1 [CB1] and type 2 [CB2] [53]. Contrary to anandamide OEA and PEA are well-known agonists of peroxisome proliferator-activated receptors (PPARs)-α[52]. The activation of these PPARs has been shown to exert a significant role in many physiological and pathological conditions such as oxidative stress[54], inflammation[55], pain[56] and memory consolidation[57]. In accord with these evidences, is the discovery that the levels of these AEAs are higher in several condition associated with redox homeostasis impairment, likely because this represents a sort of survival response

toward oxidative damage[58]. Indeed, it has been shown that OEA and PEA exert protective effects in many diseases by the inhibition of lipid peroxidation[7]. In particular, a previous paper demonstrated an involvement of these AEAs in inhibiting the lipid peroxidation in liver mitochondrial membranes of acute hypoxic animal models[60], a pathological condition associated with an increase in ROS; the authors suggested that the inhibitory effect of AEAs on lipid peroxidation depends on their ability to protect the membranes. Moreover, OEA and PEA appeared to inhibit Cu^{2+}-induced *in vitro* lipid peroxidation of plasma lipoproteins[12] and cardiac mitochondria[61]. All these evidences could contribute to explain the increase of lag-time and the decrease of ox-LDL found in this clinical trial. Finally, has been suggest an association between obesity and metabolic endotoxemia [62,63] characterized by increased serum levels of lipopolysaccharide (LPS) coming from the walls of gram-negative bacteria of the intestinal microbiota and probably deriving from alterations of the intestinal epithelium promoted by dysbiosis . LPS is an antigen that after its binding to toll-like receptor (TLR)-4 activates the inflammatory nuclear factor-kB pathway and consequently enhances oxidative stress [64]. Interestingly, both GTPs and OEA have been shown to prevent LPS-induced inflammation. Wang et al. have demonstrated that GTPs reduced LPS-induced hepatic NF-κB signaling and inflammasome activation in mice [65]. Moreover, it has been recently found that OEA pre-treatment counteract the elevation in circulating LPS in a murine model of intestinal barrier dysfunction and that this reduction is only partial under intraperitoneal treatment and almost total under oral OEA administration [66], such as in the present study. The origin of this decrease in endotoxemia could be a direct action of OEA in intestinal barrier, modulating gut inflammation, bacterial toxin translocation from the intestinal lumen to the systemic circulation and, consequently, oxidative stress [66].

In conclusion, considering that oxidative stress plays a crucial role as a critical factor linking obesity with its related complications, the evidences shown in this parallel-arm, double-blind,

placebo-controlled study demonstrate that NOPE-EGCG supplementation might enhance the preventive effect of weight loss against obesity's co-morbidities. Further studies in this area are justified and, in particular, long-term studies on subjects with higher BMI and/or exposed to cardiovascular risk factors and/or affected by oxidative stress-related pathologies are needed to reach definitive conclusions.

Conflicts of interest

None of the authors had a financial or personal interest in any company or organization sponsoring this study (CHEMI S.p.A. (Italfarmaco Group, Italy).

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Table 1. Baseline characteristics of NOPE-EGCG and placebo groups.

	(i) NOPE-EGCG (n = 71)	(ii) Placebo (n = 67)
Females/ males	53/18	53/14
Age (y)	38 ±10	41 ±11
BMI (Kg/mq)	30.5 ± 3.2	30.0 ± 3.7
Cholesterol (mmol/L)	5.31 ± 0.81	5.18 ± 0.98
HDL cholesterol (mmol/L)	1.13 ± 0.38	1.61 ± 0.38
Triacylglycerols (mmol/L)	1.15 ± 0.59	1.05 ± 0.60
Glucose (mmol/L)	5.0 ± 0.5	4.9 ± 0.6
CRP (mg/dl)	0.4 ± 0.4	0.4 ± 0.7
Lag-time of plasma peroxidation kinetic (min)	137.5 ± 18.1	138.4 ± 13.7
Slope of plasma peroxidation kinetic (F.U./min)	1.3 ± 0.2	1.4 ± 0.3
d-ROMs (mg/dL)	33.1 ± 6.7	30.5 ± 4.7
Ox-LDL (U/L)	106.4 ± 13.3	106.5 ± 15.2

Values are means ± SD. BMI: body mass index; CRP: C-reactive protein; d-ROMs: reactive oxygen metabolites; Ox-LDL: oxidized LDL.

Table 2. Treatment effects on plasma lipid peroxidation kinetic parameters (Lag-time and slope) and oxidative stress markers.

Variable Δ	*NOPE-EGCG complex mean change (95% CI)**	*Placebo mean change (95% CI)*	*T mean*
Lag-time (min)	5.38 (8.85 ÷ 1.90)	0.01 (-3.56 ÷ 3.55)	5.3
Slope (F.U./min)	0.006 (-0.05 ÷ 0.04)	0.04 (-0.01 ÷ 0.08)	0.0
d-ROMs (mg/dL)	-0.23 (-1.02 ÷ 0.55)	-0.36 (-1.09 ÷ 0.38)	-0.1
Ox-LDL (U/L)	-4.86 (-6.57 ÷ -3.14)	-1.71 (-4.29 ÷ 0.88)	3.1

*Mean changes over time and 95% CI were calculated as final value minus baseline value
d-ROMs: reactive oxygen metabolites; Ox-LDL: oxidized LDL.