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Original Contribution

Effect of dietary virgin olive oil on urinary excretion of etheno–DNA adducts

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Abstract

A significant protective effect against cancer and coronary heart disease has been attributed to the Mediterranean diet, in which olive oil is the main source of fat. Dietary antioxidants, as phenolic compounds from virgin olive oil, are candidates for reducing cancer risk by minimizing oxidatively derived DNA damage. Etheno–DNA adducts are formed as a result of oxidative stress and lipid peroxidation. To evaluate whether phenol-rich virgin olive oil influences urinary excretion of the etheno–DNA adducts εAde, εdA, and εdC as markers of oxidative stress, a randomized, double-blinded, crossover trial with three intervention periods was conducted in 28 healthy men. Each intervention was of 3 weeks' duration and separated by 2-week washout periods. Twenty-five milliliters of similar olive oils, but with differences in their phenolic content (from 2.7 to 366 mg/kg), were supplied to each subject per day. The urinary excretion of the DNA adducts was assayed by LC-MS/MS in samples before and after consumption of high phenolic content olive oil (virgin). The 24-h excretion rate did not differ significantly between baseline and after virgin olive oil consumption: εAde, 105.5 ± 40.8 vs 116.4 ± 53.4 pmol εAde/24 h ($p = 0.21$); εdA, 37.9 ± 24.8 vs 37.6 17 ± 24.2 pmol εdA/24 h ($p = 0.93$); and εdC, 218.7 ± 157.2 vs 193.5 ± 64.7 pmol εdC/24 h ($p = 0.37$). Multiple regression analysis showed a significant association between etheno–DNA adduct excretion rate and the dietary intake of linoleic acid (C18:2, ω-6) in healthy men. Consumption of 25 ml per day of phenol-rich virgin olive oil for 3 weeks did not modify to a significant degree the urinary excretion of etheno–DNA adducts in 28 healthy volunteers.

Keywords: Olive oil; Etheno adducts; Antioxidants; Urine; Polyphenols; Free radicals

There is growing epidemiological evidence which supports the fact that the Mediterranean diet has a beneficial effect on diseases associated with oxidatively generated damage, such as coronary heart disease, cardiovascular and neurodegenerative diseases, and cancer, and on aging [21]. The most common element in the dietary habits of Mediterranean populations is the consumption of olive oil. Olive oil is rich in monounsaturated fatty acids. Its main compound oleic acid is far less susceptible to lipid peroxidation than the polyunsaturated fatty acid (PUFA) linoleic acid, which predominates in sunflower oil, among others [20,23]. Oleic acid, however, may not be the only component of olive oil protecting the cell from oxidation; some types of olive oil contain a vast range of minor compounds, the most studied being the antioxidant phenolic compounds. Virgin olive oil is rich in phenolic derivatives, whereas refined olive oil loses most of these antioxidants during refining procedures. Common olive oil, also present on the market, is a mixture of refined and virgin olive oil. However, the refining procedure does not change the fatty acid composition. The protective effect of virgin olive oil against the initiation of cancer can occur by the prevention of oxidatively produced damage to DNA, including DNA strand breaks [2]. However, data from controlled intervention trials in humans on the in vivo effects of olive oil high in phenolic compounds are scarce and controversial (see latest consensus report [21]).

Exocyclic DNA adducts, e.g., 1,N^6-etheno-2′-deoxyadenosine (εdA), are formed by the reaction between DNA bases and intermediates from the lipid peroxidation of PUFAs. These etheno–DNA modifications have miscoding base-repairing

Abbreviations: AA, ascorbic acid; DHAA, dehydroascorbic acid; εdC, 3, N^4-etheno-2′-deoxycytidine; εAde, 1,N^6-ethenoadenine; εdA, 1,N^6-etheno-2′-deoxyadenosine; LA, linoleic acid; LPO, lipid peroxidation; MPA, *meta*-phosphoric acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; UA, uric acid; WBC, white blood cells.

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properties upon replication or transcription. Furthermore, they can accumulate in DNA after chronic carcinogen exposure and are considered highly mutagenetic lesions [1]. Etheno–DNA adducts are recognized by specific repair enzymes, further confirming that these genotoxic DNA lesions are recognized by cellular defense mechanisms. Thus far, the exact biological roles of the various repair systems in the repair of etheno–DNA adducts are not clear. Urinary excretion of etheno–DNA adducts could arise either from their formation in the nucleotide pool and their subsequent enzymatic hydrolysis or as the repair of damaged DNA bases in cellular DNA. The levels of etheno–DNA adducts from humans are highly variable and affected by gender, lifestyle, the dietary intake of antioxidants, and the type and amount of fatty acids [19]. It has also been shown that individuals with chronic diseases have a significantly higher level of ε–DNA adducts compared to healthy controls [7,24], hypothesized to be due to increased oxidative stress. A correlation between vitamin E and εdA in white blood cells has been demonstrated [10]. Also, a relationship between intake of ω-6 PUFA and urinary excretion of εdA has been observed [11]. Thus, these data indicate that etheno–DNA adducts may be used as a biomarker for lipid peroxidation-derived DNA damage.

Taking advantage of the available urine samples from the EUROLIVE study, a European study on the effect of olive oil on oxidatively generated damage in European populations, we have evaluated the effect of the phenol-rich virgin olive oil on urinary excretion of etheno adducts, DNA markers of oxidative stress, in healthy men. The study was designed as a randomized, double-blinded, crossover, clinical trial, during which all subjects received 25 ml daily of raw virgin, common, and refined olive oil. We evaluated the effect of the virgin olive oil by addressing the difference between baseline measurement and measurement after virgin olive oil administration.

Experimental procedures

Participants

Thirty men were recruited between June and August 2002 by advertisements in local newspapers. All potential subjects were interviewed by using a short questionnaire that sought information about their general health, family history, use of medication or nutritional supplement, and smoking status. The subjects had to meet the following criteria: living in the greater Copenhagen area, nonsmoker, age 20–60 years, not using antioxidant supplements or medication, body mass index (BMI) <32 kg/m^2, no reported chronic disease, and no history of metabolic disease. The subjects constituted a Danish subsample of the EUROLIVE study. Informed written consent was obtained from all study subjects before participation. The local ethical committee approved the protocol (No. 01-099102), and procedures were performed according to the principles of the Helsinki Declaration.

Experimental protocol (Fig. 1)

The study was designed as a randomized, double-blinded, crossover, clinical supplementation trial, during which all subjects received daily 25 ml of virgin, common, or refined olive oil containing 366, 164, and 2.7 mg/kg total phenols, respectively. The olive oil was taken raw and other crude fats were substituted for by the olive oil. Participants were randomly assigned to one of three orders of olive oil administration, using a 3×3 Latin square design. The subjects were blinded to the type of olive oil they were given. Before the first treatment period there was a run-in period of 2 weeks. Administration periods were separated by washout periods of 2 weeks in which olive oil and olives were avoided. The olive oil characteristics have been published separately by another EUROLIVE partner [4]. In summary, the olive oils given to the subjects were from the same cultivar, specially prepared for the trial from extra virgin olive oil (from Picual olives, Spain). Olive oils did not show differences in their fat and micronutrient (i.e., vitamin E, triterpenes, sitosterols) composition, with the exception of their phenolic content. The subjects' daily diets were not restricted, but the participants were advised not to consume an excessive amount of antioxidants and phenolic compounds. During intervention periods, subjects were requested to consume their usual source of raw fat for cooking purposes without any restrictions. The EUROLIVE trial has been registered in Current Controlled Trials, London, with the International Standard Randomized Controlled Trial No. ISRCTN09220811.

Anthropometric variables (height, weight, systolic and diastolic blood pressure) were recorded at all sessions. Blood pressure measurements were recorded by a mercury sphygmomanometer after a minimum of 10 min rest in the seated position; an average of two measurements was taken for analyses. All participants filled in two questionnaires at sessions 1, 3, 5, and 7. One questionnaire assessed the type and amount of food eaten in the past 3 days before the session, scales or household measures being used to gauge portion sizes wherever possible. A second questionnaire was filled in regarding the past 7-day record of alcohol consumption. Volunteers were encouraged to keep their diets, alcohol intake, and exercise patterns unchanged during the study. Dietary intakes were analyzed using the software DanKost 3000 (Dankost A/S, Herlev, DK). An outline of the study design is provided in Fig. 1.

Sampling

Venous blood samples were obtained after a 10-h fast and 24-h urine samples were collected before the randomization visit and before and after each intervention period. Blood samples were immediately centrifuged (2000*g*, 5 min, 4°C). One 0.5-ml plasma aliquot was acidified with an equal volume of 10% *meta*-phosphoric acid (MPA) containing 2 mM EDTA, briefly vortex mixed, and centrifuged (16,000*g*, 2 min, 4°C), and the supernatant was frozen at −80°C for plasma ascorbic acid (AA), dehydroascorbic acid (DHAA), and uric acid (UA) analyses. The remaining plasma aliquots were stored at −80°C. The urine samples were thoroughly mixed and aliquots were stored at −20°C before analysis.

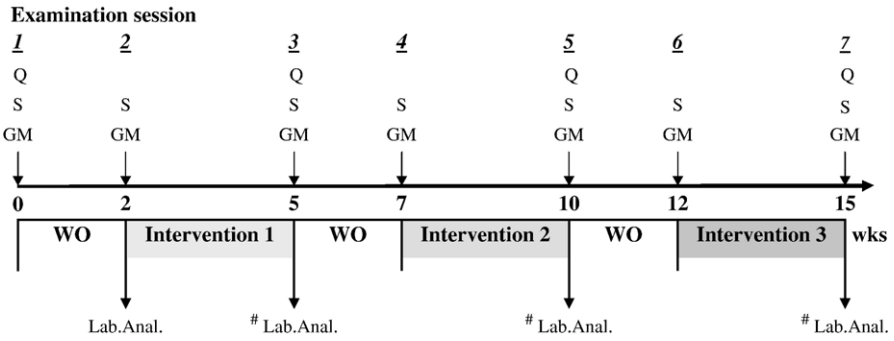

Fig. 1. Study design. Q, questionnaires; S, sampling of blood and 24-h urine; GM, general measurements (weight, blood pressure). WO, washout period. Lab.Anal., laboratory analyses of εdA, AA, DHAA, and UA). # indicates that analyses were performed only on samples of participants to which virgin olive oil was administered.

Laboratory analyses

The concentrations of AA and UA in MPA-stabilized serum samples were analyzed by HPLC with coulometric detection as previously described [14]. The 1,N^6-ethenoadenine (εAde), εdA, and 3,N^4-etheno-2′-deoxycytidine (εdC) concentrations were measured in 24-h urine samples by a column-switching LC/APCI-MS/MS assay as previously described [13].

Markers of compliance

Tyrosol and hydroxytyrosol, the two major phenolic compounds present in olive oil as simple forms or conjugates [20], were measured in 24-h urine before and after each intervention period as biomarkers of compliance of the type of olive oil ingested.

Statistical analysis

All results are reported as the means ± standard deviation. Statistical analysis of the two groups was performed using Student's paired *t* test, and $p < 0.05$ was considered significant. Simple relationship among variables was assessed by means of Pearson's correlation. A general linear regression model (backward stepwise), with the administration order as covariate, was employed to evaluate the relationship between etheno–DNA adduct excretion and the different independent variables from the dietary records. STATISTICA version 7.0 (StatSoft, Inc., Tulsa, OK, USA) was used for these analyses.

Results

Subject characteristics

Of the 30 subjects who started the protocol, 2 withdrew during the study. One withdrew due to a dislike of the test products. The other withdrew for personal reasons. The baseline characteristics of subjects who completed the study are provided in Table 1. No changes in diet, weight, or lifestyle were found from the time of recruitment to the completion of the study.

Markers of compliance

There was significantly increased urinary excretion of tyrosol (mean value from 271 to 614 μg/L, $p = 0.002$) and hydroxytyrosol (mean value from 361 to 1591 μg/L, $p < 0.001$)

Table 1
Subject characteristics at baseline

	All subjects ($n = 28$)	Order 1 ($n = 10$)	Order 2 ($n = 9$)	Order 3 ($n = 9$)
Age (years)	32.1 ± 9.7	32.0 ± 9.1	34.3 ± 11.1	30.1 ± 9.5
Height (cm)	181 ± 6.8	184 ± 5.6	179 ± 7.4	180 ± 7.0
Weight (kg)	76.6 ± 10.3	80.1 ± 11.3	72.7 ± 9.4	76.7 ± 9.6
Body mass index (kg/m^2)	23.3 ± 2.5	23.6 ± 2.5	22.7 ± 2.5	23.7 ± 2.6
Waist circumference (cm)	81.9 ± 8.7	83.0 ± 11.0	79.7 ± 7.3	82.9 ± 7.7
Hip circumference (cm)	94.0 ± 7.6	98.0 ± 9.4	91.4 ± 5.6	92.3 ± 5.9
Waist–hip ratio	0.87 ± 0.08	0.85 ± 0.09	0.87 ± 0.07	0.90 ± 0.08
Systolic blood pressure (mm Hg)	130 ± 10	132 ± 8	134 ± 11	124 ± 9
Diastolic blood pressure (mm Hg)	78 ± 6	78 ± 4	80 ± 9	75 ± 4
Energy (MJ/day)	9890 ± 2173	9329 ± 2197	9980 ± 2040	10,423 ± 2374
Protein (g/day)	84.5 ± 22.0	82.7 ± 20.4	84.1 ± 24.4	86.9 ± 23.6
SAFA (g/day)	35.5 ± 15.2	34.2 ± 18.5	29.0 ± 10.6	43.5 ± 12.7
MUFA (g/day)	26.9 ± 10.6	27.1 ± 11.1	21.1 ± 7.0	32.4 ± 11.0
PUFA (g/day)	9.9 ± 4.0	10.0 ± 2.9	8.1 ± 3.5	11.4 ± 5.0
Carbohydrate (g/day)	297.2 ± 72.9	280.4 ± 65.5	303.7 ± 73.4	309.4 ± 84.7

Values are expressed as means ± SD. Order 1, virgin, common, and refined olive oil; Order 2, common, refined, and virgin olive oil; Order 3, refined, virgin, and common olive oil.

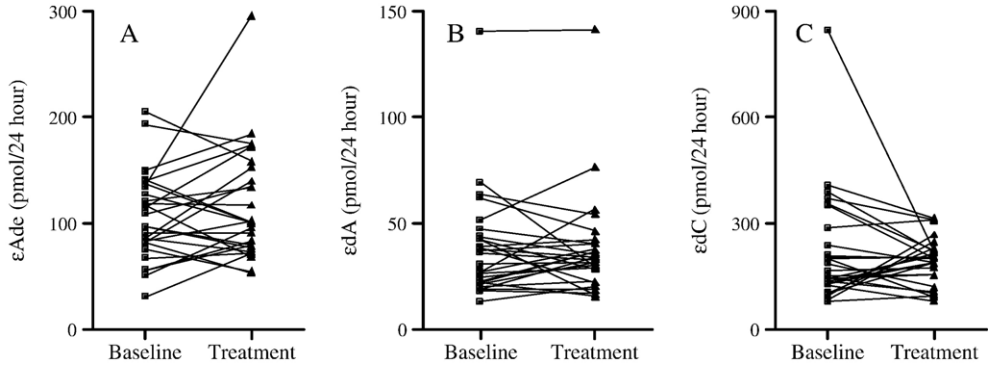

Fig. 2. Line plot of the etheno–DNA adduct excretion rate for baseline–virgin olive oil treatment for each subject. (A) εAde, (B) εdA, and (C) εdC.

between baseline and treatment, indicating adherence to the protocol.

Effect of treatment

There was no effect of the 3-week dietary virgin olive oil intervention on the urinary excretion of εAde (105.5 ± 40.8 vs 116.4 ± 53.4 pmol εAde/24 h, $p = 0.21$), εdA (37.9 ± 24.8 vs 37.6 ± 24.2 pmol εdA/24 h, $p = 0.93$), or εdC (218.7 ± 157.2 vs 193.5 ± 64.7 pmol εdC/24 h, $p = 0.37$). Furthermore, no significant differences were observed among groups of order of oil administration. Fig. 2 shows the εdA excretion rate at baseline from examination session 2 and after the virgin olive oil administration period.

Correlations

As a hypothesis-generating approach, an evaluation of which factors best predicted the three different etheno adduct possible relationships was done by correlation analysis. There was no significant correlation of the individual etheno–DNA adducts (e.g., εAde, εdA, and εdC) with age, weight, blood pressure, or BMI. There was no significant correlation between etheno adduct excretion and concentration of any of the plasma variables, AA, DHAA, UA, or vitamin E, after the 3-week olive oil intervention. Pearson's correlation between etheno–DNA adduct excretion rate and dietary intake variables yielded the significant associations shown in Table 2. These univariate associations were further explored in a backward stepwise multiple regression analysis for the individual etheno nucleoside adduct (e.g., εdA and εdC) as a dependent variable. Dietary intake of linoleic acid was found to significantly predict urinary excretion of εAde, εdA, and εdC. Dietary intake of linoleic acid (regression coefficient 0.686, SE 0.143; $p = 0.00006$) was found to be the only factor significantly predicting urinary εdA excretion (adjusted $R^2 = 0.45$, $p = 0.00006$), whereas dietary intake of linoleic acid (regression coefficient 0.515, SE 0.141; $p = 0.0012$) and iron (regression coefficient 0.522, SE 0.141; $p = 0.0011$) were found to significantly predict urinary εdC excretion (adjusted $R^2 = 0.46$, $p = 0.00016$).

Discussion

The relative contributions of various fatty acids to the dietary intake differ widely among countries, as do the incidence of many cancers. Previous studies in humans have shown that olive oil, compared with seed oils, has the ability to prevent lipid peroxidation due to its high MUFA content [16]. It is only recently that investigations have addressed the influence of antioxidants from virgin olive oil on oxidative status in humans [6,8,15,17,21,22,26–29]. Etheno–DNA adducts are generated endogenously by the reaction of DNA with the major lipid peroxidation (LPO) product, *trans*-4-hydroxy-2-nonenal. Significantly elevated levels of these adducts have been found in organs with diseases related to persistent inflammatory processes that can lead to malignancies [3]. The importance of endogenously induced DNA lesions is also indicated by the multiple specific DNA repair systems that are highly efficient in the repair of etheno lesions [9]. Thus, etheno–DNA adducts may qualify as potential markers of risk prediction and as targets for preventive measures. To our knowledge, until now, no in vivo studies have investigated the effect of phenol-rich olive oil consumption on etheno–DNA adduct excretion.

We did not find an effect of virgin olive oil consumption on the urinary excretion of etheno–DNA adducts. The average excretion rate of εdA in our study was similar to values found by others [11,12,18]. The reason for the lack of significant differences between the two periods may be that both periods

Table 2
Univariate analysis of the relationship between etheno–DNA adduct excretion rate and potentially explanatory dietary intake variables[a]

	Palmitic acid (C16:0)	Linoleic acid (ω-6; C18:2)	Linolenic acid (ω-3; C18:3)	Cholesterol	Vitamin C	Iron
εAde		0.399 (0.039)				
εdA		0.687 (0.00006)	0.567 (0.002)			
εdC	0.408 (0.035)	0.483 (0.011)	0.448 (0.035)	0.449 (0.019)	0.422 (0.028)	0.485 (0.010)

[a] Pearson's correlation coefficient: r (p value). εAde, 1,N^6-ethenoadenine; εdA, 1,N^6-etheno-2′-deoxyadenosine; εdC, 3,N^4-etheno-2′-deoxycytidine.

were very similar with regard to the intake of energy derived from fat, as well as the dietary fatty acid pattern. The daily dose (25 ml) of raw virgin olive oil consumed in this study was comparable with that of other studies [20,23] and within the range of current intake in the Mediterranean region. However, it is possible that the background diet of our subjects already contained sufficient antioxidants to mask any additional antioxidant effects from the virgin olive oil.

Concerning in vivo human oxidatively generated damage, a decrease in the urinary excretion rate of a specific marker of oxidative stress, 8-oxodG, was reported after short-term consumption of olive oil high in phenolic compounds [29]. In that study [29] participants were subjected to a very low antioxidant diet 3 days before and during the intervention periods, so it was rather a depletion study and not a supplementation study. Regarding lipid peroxidation, Visioli et al. found a dose-dependent decrease in isoprostane excretion after a single administration of four different olive oils highly enriched in phenolic compounds to six males [25]. Marrugat et al. found a dose-dependent decrease in in vivo oxidized LDL with the phenolic content of the olive oil administered [15]. Such an effect was not observed in fasting blood samples in three other human studies investigating the effect of virgin olive oil phenols on other lipid oxidation markers [17,27,28]. There are two major differences between the study by Marrugat et al. and our study. First, refined olive oil was used as the source of crude fat in washout periods and for cooking purposes during washout and intervention periods. Second, the effect was assessed as changes between the beginning and the end of each intervention. However, because there were no differences in diet between intervention periods of this study, it is not likely that the differences mentioned should explain our findings. In the study of Marrugat et al., differences in LDL oxidation were low. If changes in LPO after olive oil consumption were small this could be a reason εdA changes, as a product of the reaction between DNA bases and LPO intermediates, were not detected in our study.

Our results indicate that the lipid peroxidation-derived DNA damage markers εAde, εdA, and εdC were not affected to a meaningful degree by age, weight, or BMI. Of the nutrients reported by the questionnaire, several dietary variables correlated with the etheno–DNA adducts. However, due to colinearity of variables calculated from the questionnaire, stepwise general linear regression models were obtained with only linoleic acid (LA) resulting in a significant and meaningful correlation with the excretion rate of the etheno–DNA adducts. The associations between the etheno–DNA adducts and dietary factors have been observed in several studies. In a study comparing a diet high in ω-6 PUFA or MUFA, Nair et al. found highly elevated concentrations of εdC and εdA in the WBC of females on a PUFA diet, whereas the levels in males were similar for both groups [19]. However, in a larger study on healthy female volunteers, no correlation of the levels of WBC adducts with either high LA daily intake or LA serum concentration was found [10]. In both studies a large intrasubject variation among females was observed that the authors ascribed to variations in hormonal metabolism. Hanoaka et al. conducted a multiple regression and found a correlation between εdA excreted in the urine of postmenopausal women and ω-6 PUFA intake together with NaCl excretion [11]. In a rat model, the effects of a vegetable-oil-supplemented diet (sunflower, rapeseed, olive, or coconut oil) on oxidative stress-related DNA adducts in the liver were investigated [5]. No correlations between ω-6 linoleic acid content in the four diet groups were found for levels of either εdA or εdC. Furthermore, no protective influence on the formation of the etheno–DNA adducts by the ω-3 linolenic acid or olive oil antioxidant was found.

In conclusion, the 24-h excretion rate of etheno–DNA adducts did not differ significantly between baseline and after consumption of virgin olive oil with the highest phenolic content (150 mg/kg). Multiple regression analyses found a significant association between etheno–DNA adduct excretion rate and the dietary intake of linoleic acid (C18:2, ω-6) in healthy men.

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