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Dietary extra-virgin olive oil rich in phenolic antioxidants and the aging process: long-term effects in the rat☆

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Abstract

The aim of the present work was to verify whether extra-virgin olive oil, a food naturally containing phenolic antioxidants, has the potential to protect from the pro-aging effects of a high-calorie diet. Male rats were fed from age 12 months to senescence a high-calorie diet containing either corn oil (CO), or extra-virgin olive oil with high (H-EVOO) or low (L-EVOO) amounts of phenols. The prolonged high fat intake led to obesity, liver lipid degeneration and insulin resistance, which were not counteracted by high phenol intake. No difference in overall survival was found at the end of the experiment in the animals treated with H-EVOO compared to the other groups. However, we did detect a protective effect of olive oil on some age-related pathologies and on blood pressure, of which the former was associated with the antioxidant content. Concomitantly, a decrease in DNA oxidative damage in blood cells and plasma TBARS and an increase in liver superoxide dismutase were detected following H-EVOO consumption. Thus, although olive oil phenols cannot reverse the detrimental effects of a prolonged intake of high amounts of fat, improving the quality of olive oil in terms of antioxidant content can be beneficial.

Keywords: Fatty acids; High calorie diet; Liver steatosis; Olive oil phenols; Oxidative damage

1. Introduction

Age-related diseases are an increasing problem in the Western world due to the longer average lifespan of the human population. The free radical theory of aging has provided one of the suggested mechanisms to explain this condition. In fact, under some experimental conditions, it has been shown that reducing free radical production can significantly increase lifespan [1]. It is also known that aging is under genetic control: knocking out certain genes, such as those forming the insulin–IGF-1 (insulin-like growth factor 1) pathway, can increase lifespan in different organisms including mammals [2,3], whereas genes like the FOXO (Forkhead box O) transcription factor family have to be activated in order to obtain antiaging effects [4,5]. The free radical and genetic theories on aging do not exclude each other and might actually concur to produce a more refined description of a complex phenomenon. In fact, the products of some of the genes involved in aging control are actually related to oxidative stress management, such as the FOXO transcription factors [6] and the p66Shc redox protein, whose ablation through targeted mutation increased lifespan and oxidative stress resistance in mice [7].

All these observations have suggested the possibility of modulating age-related genes via pharmacological [8] or dietary interventions. Among the latter, caloric restriction has, up to now, been the only intervention able to consistently and substantially increase lifespan in all organisms, from yeast to primates [9]. Unfortunately, the average daily caloric intake of people living in developed countries has progressively increased during the last century and is presently over 3000 kcal (3750 in the United States), whereas the recommended intakes are around 1900–2200 kcal [10]. Epidemiological studies have reported that excess caloric intake is associated with increased incidence of several chronic age-related diseases, such as obesity, diabetes, cancer, cardiovascular disease as well as neurodegenerative disorders [11,12]. An alternative way which is being actively explored to delay age-related diseases without calorie restriction is the use of the so-called calorie restriction mimetics [13].

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One of these molecules is resveratrol, a natural compound produced by grapes, which has been shown to increase lifespan and to modulate various age-related genes in mammals who consume it at high doses, and which cannot be provided by any natural food [14]. Resveratrol is currently being studied in clinical trials and is already used as a food integrator.

The aim of the present work was to verify whether a food naturally containing high amounts of antioxidants has the potential to protect from the proaging effects of a high calorie diet. Olive oil is a typical component of the Mediterranean diet, containing variable amounts of phenolic antioxidants [15]. We selected an Italian olive oil rich in antioxidants and administered it to rats from middle to advanced age. The oil was mixed with the animal diet to reach a final fat proportion of 23% (w/w, dry diet), corresponding to about 44% of the calories provided by the whole diet, in line with the fat intake associated with the Western Europe and North America diet [16]. As a control, olive oil depleted of most phenolic antioxidants but identical to the oil in study for all the other major and minor components was used. We also used corn oil as a further control to check for the effects of different fatty acids on the aging process.

2. Methods and materials

2.1. Animals and treatments

All procedures were carried out in agreement with the European Union Regulations on the Care and Use of Laboratory Animals (Official Journal of European Compliance Laboratory 358/1, 12/18/1986) and the experiments were conducted according to Italian regulations on the protection of animals used for experimental and other scientific purposes (Decreto Ministeriale 116/1992), after approval from the Italian Ministry for Scientific Research.

Male Wistar rats aged 10 months at the beginning of the experiment were used. The animals were randomly subdivided into three groups of 20 rats each and fed with different diets (15 g per rat per day) for 12 months. The experimental diets were prepared using components purchased from Piccioni (Gessate, Milan, Italy). Fresh diet was put in the trough every day. We used a modified high-fat AIN 76 diet (lipids providing about 40% of the total calories), composed of (g/100g of diet): 32.2 sucrose, 23.0 fat, 23.0 casein, 10.1 corn starch, 6.0 cellulose, 4.0 AIN 76 mineral mix, 1.2 AIN 76 vitamin mix, 0.3 methionine and 0.2 choline.

One group of rats was fed a diet in which the lipid component was provided by corn oil (CO), another group by an extra-virgin olive oil high in natural antioxidants (H-EVOO), and the third group by the same extra-virgin olive oil deprived of phenolic compounds (L-EVOO). Corn oil was purchased from Tampieri (Faenza, Ravenna, Italy), while extra-virgin olive oil (H-EVOO) was purchased from Cipolloni and Petesse (Foligno, Perugia, Italy). The L-EVOO was obtained from H-EVOO as follows: the extra-virgin oil was homogenized for 1 min with water (1:1, v/v), and the oil was separated by centrifugation (Westfalia separator); this procedure was repeated six times. Then the oil was filtered through a cellulose acetate membrane.

During the 12-month treatment with the different oils, the animals were checked for their general health status everyday, and body weight was measured every 2 weeks. The animals that died or had to be euthanized during the treatment, as well the animals sacrificed at the end of the treatment, were autopsied, and organ pathologies were assessed. After decapitation, blood was partly collected by decantation into anticoagulated tubes (containing sodium citrate) and centrifuged at 1000×*g* for 10 min to separate plasma from erythrocytes. Plasma was stored at −80°C. Part of the blood was collected in nonanticoagulated tubes for serum analysis. The liver was removed from the sacrificed animals and weighed. Part of the tissues was used immediately to measure the endogenous levels of DNA damage (see below), part was stored at −80°C and part was fixed in formaldehyde for histological analysis (hematoxylin and eosin staining).

2.2. Extraction and high-performance liquid chromatography analysis of phenolic compounds of the oils

The extraction of phenols and the high-performance liquid chromatography (HPLC) analysis were conducted as reported [17] using an Agilent Technologies system model 1100 (Agilent Technologies, Palo Alto, CA, USA), composed of a vacuum degasser, a quaternary pump, an autosampler, a thermostated column compartment, a diode array detector and a fluorescence detector (FLD). The analysis of the oil extract was performed using C18 columns Spherisorb ODS-1 250×4.6 mm with a particle size of 5 μm (Phase Separation, Deeside, UK). The mobile phase was composed of 0.2% acetic acid (pH 3.1) in water (solvent A)/methanol (solvent B) at a flow rate of 1 ml/min, and the gradient changed as follows: 95% A/5% B for 2 min, 75% A/25% B for 8 min, 60% A/40% B for 10 min, 50% A/50% B for 16 min and 0% A/100% B for 14 min, and this composition was maintained for 10 min. Then, the initial conditions were reset, and equilibrium was reached for 13 min; the total running time was 73 min.

2.3. Extraction and HPLC analysis of α-tocopherol from the oils

The α-tocopherol was analyzed as described [18]: 2 g of oil was dissolved in 10 ml of hexane and then filtered through a PVDF (polyvinylidene fluoride) syringe filter (0.2 μm) and injected into the HPLC system through a Waters μPorasil column (300 mm×3.9 mm×10 μm, Milford, MA, USA) using a 100% step mobile phase gradient of n-hexane with 0.5% isopropylic alcohol (A)/0% n-hexane with 10% isopropylic alcohol (B) for 4 min, 60% A/ 40% B for 14 min, 40% A/60% B for 4 min and 100% A/0% B for 3 min. This last gradient is maintained for 5 min with a flow rate of 2 ml/min. The total running time is 30 min and the injection volume 50 μl. The detector was an FLD operated at an excitation wavelength set at 294 nm, and the emission, at 330 nm.

2.4. Measurement of tissue oxidative damage levels

2.4.1. Comet assay

DNA damage in whole blood and liver was evaluated by the comet assay, as previously described [19,20].

Briefly, aliquots of whole blood and liver homogenates were suspended in agarose and layered on microscopic slides to be run through the comet assay. After a lysis step, a bacterial repair enzyme, formamidopyrimidine glycosylase (FPG), which introduces breaks at purine oxidation sites, was used to determine oxidized DNA bases. Microscopic analysis was carried out using a Labophot-2 microscope (Nikon, Japan) provided with epifluorescence. Each experimental point was run in duplicate, and the images of 50 randomly chosen nuclei per slide were captured and analyzed using a custom-made imaging software coupled with a CCD camera (model C5985, Hamamatsu, Japan). The program calculated the total fluorescence distribution along the longer axis of the nucleus and the fluorescence distribution of the head and tail of the comet, respectively, for each image. Data were expressed as percentage of total fluorescence migrated to the tail of each nucleus (% DNA in tail), a parameter linearly related to the number of DNA breaks. The mean % DNA in tail of 50 nuclei per gel was calculated, and the duplicate values were further averaged. The value of % DNA in tail obtained without FPG incubation estimated the basal number of DNA strand breaks, whereas specific oxidative damage on purines (FPG-sensitive sites) was assessed by subtracting the value found in buffer-incubated slides from that obtained with FPG.

2.4.2. Thiobarbituric acid-reactive substance determination

Thiobarbituric acid-reactive substance (TBARS) concentrations in plasma and liver were quantified spectrophotometrically. A previously described method [21] was used for the plasma samples to prevent the interference of hemoglobin and its derivatives (maximum absorbance at 540 nm) during the measurement of TBARS (maximum absorbance at 532 nm). A TBA reagent prepared prior to determination consisted of 40.5 ml 20% acetic acid buffered to pH 3.5 with 1 N NaOH 13.2 ml, 8.2% SDS, and 40.5 ml 0.8% TBA, made up with twice distilled water to 100 ml. A plasma aliquot of 100 μl was mixed with 900 μl of TBA reagent, incubated at 90°C for 80 min and cooled on ice. The sample was then extracted with a solution prepared with N-butanol, twice distilled water, and pyridine (15:3:1, v/v), and centrifuged at 3000×*g* for 15 min. Absorbance readings were taken on the spectrophotometer at 510, 532, and 560 nm against a blank. In the liver, TBARS were assayed using 25 mg of frozen liver tissue, homogenized in 250 μl of RIPA buffer (SIGMA, Milan, Italy) with protease inhibitor cocktail (SIGMA). A volume of 100 μl of this homogenate was used for TBARS determination. The actual TBARS absorbance was calculated from the equation:

$$\text{Actual } A_{532} = 1.22\ [(A_{532}) - (0.56) \times (A_{510}) + (0.44) \times (A_{560})]$$

The TBARS concentration was read from a calibration curve made with 1,1,3,3-tetramethoxypropane. All samples were analyzed in duplicate, the mean value of which was taken as the final result.

2.5. Determination of superoxide dismutase and xanthine oxidase activity

For enzyme activity measurement we followed a previously described procedure [22]. About 100 mg of liver were homogenized in 1 ml of phosphate-buffered saline (PBS) and centrifuged at 1500×*g* for 15 min at 4°C. A fraction of 100 μl of the supernatant was used to determine superoxide dismutase (SOD) activity by nitro blue tetrazolium (NBT) reaction: 1.2 ml of 50 mM Na_2CO_3 (pH 10.2), 50 μl of 3 mM EDTA, 50 μl of 3 mM xanthine, 50 μl of 0.8 mM NBT and 50 μl of xanthine oxidase (XO) (140 mU/ml initial concentration) were added to each sample. Samples were incubated for 30 min at 2°C. The inhibition of NBT reduction in each sample was determined spectrophotometrically at 560 nm. SOD activity, expressed as mU/mg protein, was evaluated by referring to inhibition brought about by the standard SOD measured at the same time.

A further 100-μl fraction of the supernatant was used for assaying XO activity. Briefly, 1.3 ml of 50 mM PBS (pH 7.4), 50 μl of 3 mM EDTA and 50 μl of 3 mM xanthine were added to each sample, and the solution was incubated for 10 min at 24°C. XO activity was assayed as uric acid production, based on the increase in absorbance at 286 nm. The enzyme activity was expressed as mU/mg protein, where one unit corresponds to the formation of 1 μmol uric acid per min.

Protein content in the homogenates was estimated by the Bio-Rad DC protein assay kit (Bio-Rad, Segrate, Milan, Italy).

Table 1
Antioxidant composition of the study olive oils (mg/kg)

Components	H-EVOO	L-EVOO
3,4-DHPEA	15.0±0.5	-
p-HPEA	11.0±0.1	0.1±0.01
3,4-DHPEA-EDA	346.7±3.5	1.1±0.02
p-HPEA-EDA	81.9±1.1	1.7±0.01
(+)-1-Acetoxipinoresinol	17.0±0.02	1.5±0.01
(+)-Pinoresinol	32.3±0.1	2.0±0.02
3,4-DHPEA-EA	214.9±2.1	2.9±0.03
α-Tocopherol	131.2±1.1	129.1±1.10

The phenol content is the mean value of three independent determinations±S.D. 3,4-DHPEA, hydroxytyrosol; p-HPEA, tyrosol; 3,4-DHPEA-EDA, 3,4 dialdehyde form of elenoic acid linked to hydroxytyrosol; p-HPEA-EDA (oleocanthal), dialdehyde form of elenoic acid linked to tyrosol; 3,4-DHPEA-EA, hydroxytyrosol esters of elenoic acid or oleuropein.

2.6. Measurement of blood pressure and plasma/serum biomarkers

Systolic and diastolic blood pressure and cardiac frequency of the rats were measured using the tail-cuff method with BP-2000 Blood Pressure Analysis System coupled with BP-2000 analysis software (Visitech Systems, Apex, NC, USA). The measurement was done three times during the treatment. The rats were restrained in a plastic cage for approximately 15–30 min for acclimation and were minimally warmed before blood pressure determination. Five to ten stable readings of blood pressure were taken for each animal. The final measurement was the mean of the stable recordings.

Plasma insulin was measured in frozen samples using the Ultrasensitive Rat Insulin ELISA kit (Mercodia, Uppsala, Sweden). Serum was separated from blood samples and transferred to the General Laboratory of the Azienda Ospedaliero-Universitaria Careggi (Florence, Italy), where all laboratory determinations were performed using an automated assay with ADVIA 2400 and Centaur XP instruments (Siemens Medical Solutions Diagnostics, Milan, Italy).

2.7. Insulin-stimulated 2-deoxy-D-glucose uptake by adipocytes

Adipocytes from epididymal fat were obtained by collagenase digestion and then 2-deoxy-D-glucose uptake was performed as described [23], with some modifications. Aliquots of 2.5×10^4 cells were distributed in Eppendorf tubes containing 1% bovine serum albumin in glucose-free Krebs–Ringer bicarbonate, pH 7.4 (reaction volume 495 μl). A 10-min preincubation, at 37°C in 5% CO_2 atmosphere was performed before the addition of insulin stimuli. After preincubation, 2.5 μl of insulin solution were added to the reaction medium to reach concentrations from 0.5×10^{-9} to 500×10^{-9} M and cells were incubated for 30 min before the addition of 2.5 μl of 2-deoxy-D-[1,2-^{3}H]glucose (100 μM; 111 kBq). 2-Deoxyglucose uptake was evaluated 10 min after the addition of labelled deoxyglucose. The reaction was blocked by cooling samples on ice. Diisononyl phthalate 1 ml was added to each sample, vortexed and centrifuged at 4500×*g* for 10 min. The upper phase (900 μl) was added to 4 ml of scintillation counting liquid (InstaGelPlus, Perkin-Elmer, Salem, MA, USA). Radioactivity was measured in a liquid scintillation counter (1900 TR, Canberra Packard, Groningen, The Netherlands).

2.8. Statistics

Unless specifically indicated, all data were analyzed using one-way analysis of variance for parametric data (P level fixed at .05). Correlations were performed using linear regression analysis, with the significance level set at $P<.05$. Values were expressed as the mean±S.E.

To estimate animal survival, Kaplan–Meier curves were plotted, and the Log-rank test was used to compare the survival functions among groups. A test for proportion was performed to evaluate the difference in survival among the three groups at discrete times during the treatment.

3. Results

3.1. Antioxidant content of the oils

The antioxidant composition of the olive oils employed is shown in Table 1. It can be seen that the phenolic compounds were efficiently removed from H-EVOO, being much reduced in the L-EVOO. The procedure employed to produce the L-EVOO (see Methods), at variance with a refinement process, has been developed to eliminate selectively hydrophilic substances, such as phenolic compounds, leaving unmodified the other olive oil components such as tocopherols (see Table 1), squalene, sterols, fatty acids. Both the levels of phenols and tocopherols were below detection limits in corn oil.

3.2. Animal body weight

Fig. 1 shows the increase in rats' body weight during the 12-month treatment period. The animals steadily gained weight until around Week 36 and then reached a plateau. During the last weeks, the growth curves became more irregular due to an increased frequency of age-associated pathologies. A statistically significant difference was found between H-EVOO and CO groups at 50, 52 and 54 weeks of treatment. An evaluation of the amount of food left was done between Weeks 35 and 40 of treatment, and the average of 10 measurements in different days showed that the CO diet left in the trough was 1.4±0.1 g per rat per day, whereas the values for H-EVOO and L-EVOO were 0.7±0.1 and 0.8±0.1 ($P<.01$ vs. CO). However, the body weight curves were not separated at this time.

3.3. Survival

Fig. 2 shows animal Kaplan–Meier survival function during the treatment period. The log-rank test for the equality of the survival functions gave a non significant test statistics [$\chi^2(2)=0.24$], meaning

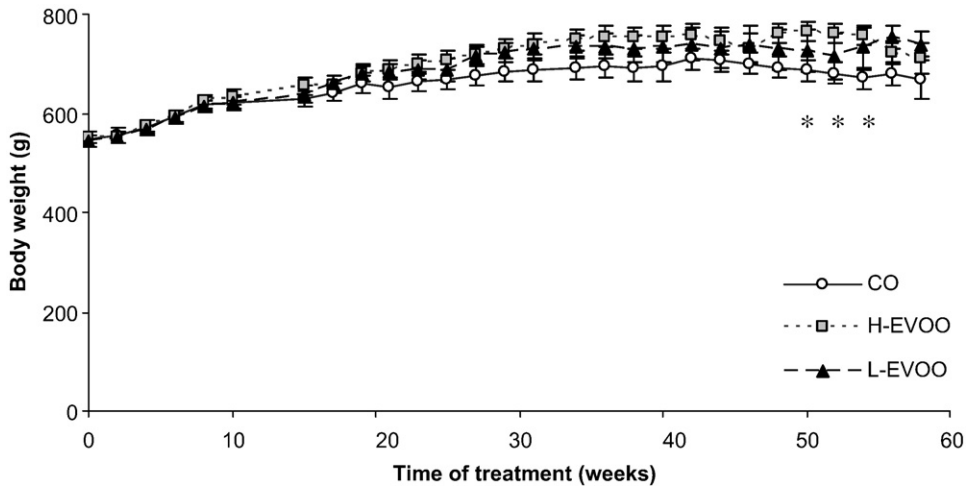

Fig. 1. Body weights of rats fed with experimental diets in which the lipid component (23% w/w, dry diet) was provided by CO, H-EVOO and L-EVOO. *$P<.05$, CO vs. H-EVOO.

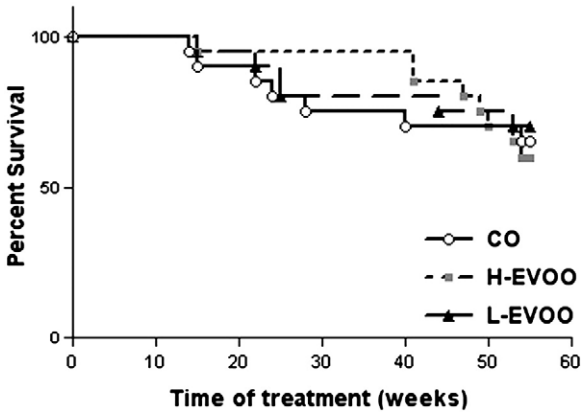

Fig. 2. Kaplan–Meier survival curves of rats fed 23% (w/w, dry diet) corn oil (CO); extra-virgin olive oil with high (H-EVOO) or low (L-EVOO) phenol content.

that there was no statistically significant difference in the overall survival of the animals in the three groups. However, the H-EVOO curve and the others separated around the 25th week, and the distance was maximal at treatment Week 40, when only one animal from the H-EVOO had died, whereas four animals from the L-EVOO and five from the CO group had died or had to be euthanized. Thus, we tested the proportion of surviving animals in the H-EVOO group compared to the others at Week 40. The proportions to be compared were 0.049 (90% CI −0.030 to 0.130) for H-EVOO and 0.097 (90% CI 0.091 to 0.409) for the others. The obtained χ^2 P value was .076.

3.4. Tissue oxidative damage

The endogenous levels of DNA breaks, FPG sites and TBARS in the liver were not different among the treatment groups (Table 2). However, a significant increase in SOD activity (about +60%) and decrease in XO activity (about −25%) were found in the H-EVOO group compared to both CO and L-EVOO.

The levels of DNA oxidized bases (FPG sites) in circulating leukocytes were lower in H-EVOO compared to CO and L-EVOO (Table 3), although the difference was significant only vs. L-EVOO. The in vitro-induced DNA damage in blood leukocytes was not different among groups (data not shown). Plasma TBARS were statistically significantly lower in the H-EVOO group compared to the other groups (Table 3).

3.5. Organ pathology

The main pathology observed was liver steatosis. Both macroscopic and microscopic analysis indicated that this was more severe in the olive oil-fed animals than in the corn oil group (Fig. 3). As shown in Table 2, the liver weight was increased in the H-EVOO group (+26%, P<.01) and in the L-EVOO group (+22%, P<.05) compared to CO. Also, the ratio between liver weight and body weight was increased in the H-EVOO group and in the L-EVOO group (P<.05) compared to CO. The increase in liver weight was positively correlated with the increase in liver triglyceride content (R=0.46, P<.01) which was also significantly increased in the H-EVOO and L-EVOO group compared to CO (+49 and +31% respectively).

Autopsy results also showed an increase in hypophyseal tumors and ulcerative dermatitis with aging. In the H-EVOO group, a lower incidence of both these pathologies was observed (2/19 hypophyseal tumors vs. 5/15 in the CO and 5/16 L-EVOO group; 3/19 ulcerative dermatitis vs. 8/15 in the CO and 5/16 in the L-EVOO group). These differences were analyzed by means of the χ^2 test, which showed a significant difference in the incidence of ulcerative dermatitis between H-EVOO and CO.

Table 2
Liver steatosis- and oxidative damage-related parameters

	CO	H-EVOO	L-EVOO
liver weight (g)	13.59±0.47	17.13±0.79 *	16.60±1.26 *
liver weight/body weight	0.021±0.001	0.024±0.001 **	0.024±0.001 *
triglycerides (mg/g wet tissue)	45.40±3.37	66.05±8.85 *	58.95±6.81 *
DNA strand breaks (%DNA in tail)	5.78±0.58	6.65±0.91	5.97±0.94
DNA FPG sites (%DNA in tail)	18.26±2.44	16.88±2.28	17.75±1.92
TBARS (nmol/g wet tissue)	100.7±14.68	134.5±16.72	110.5±13.86
SOD activity (mU/mg protein)	241.48±38.65	390.39±43.11 *,#	248.22±36.72
XO activity (mU/mg protein)	172.36±16.31	130.02±11.39 *,#	184.45±13.41

CO, corn oil; H-EVOO, extra-virgin olive oil rich in natural antioxidants; L-EVOO, extra-virgin olive oil poor in natural antioxidants.
Values are expressed as mean±S.E.M. (n=11–13). DNA strand breaks and FPG sites were measured with the comet assay.
* P<.05 vs. CO.
** P<.01 vs. CO.#P<.05 vs. L-EVOO.

Table 3
Blood oxidative damage-related parameters

	CO	H-EVOO	L-EVOO
DNA strand breaks (%DNA in tail)	6.33±0.40	5.75±0.44	6.08±0.47
DNA FPG sites (%DNA in tail)	16.06±1.70	13.59±1.47 #	19.83±1.40
plasma TBARS (μmol/ml)	3.12±0.64	1.61±0.30 *,#	2.95±0.55

CO, corn oil; H-EVOO, extra-virgin olive oil rich in natural antioxidants; L-EVOO, extra-virgin olive oil poor in natural antioxidants. Values are expressed as mean±S.E.M. (n=11–13). Leukocyte DNA strand breaks and FPG sites were measured with the comet assay.
* P<.05 vs. CO.
# P<.05 vs. L-EVOO.

3.6. Plasma/serum parameters and blood pressure

The measured plasma and serum biomarkers are shown in Table 4. Cholesterol and amylase levels were significantly increased in H-EVOO and L-EVOO groups. A tendency towards an increase in triglyceride and insulin levels was also observed in the same groups, which, however, was not statistically significant.

Blood pressure and cardiac frequency were measured three times during the treatment. CO rats showed an increase in systolic blood pressure at the end of the treatment, which was not present in the olive oil-treated animals (P<.05, Fig. 4). No time-or group-related difference was found in diastolic blood pressure or cardiac frequency (data not shown).

3.7. Insulin sensitivity in epididymal fat

Epididymal fat cells prepared from CO animals showed insulin-dependent glucose uptake (Fig. 5). Instead, in the epididymal fat cells prepared from the L-EVOO and H-EVOO animals, glucose uptake did not increase upon insulin exposure, indicating a state of insulin resistance in this tissue.

4. Discussion

The aim of this study was to verify whether phenolic antioxidants in olive oil have the potential to counteract the effects of a high fat diet during aging. Among this group of phenols, hydroxytyrosol (3,4-DHPEA) and its derivatives are mainly responsible for the antioxidant activity of extra-virgin olive oil [24]. Orally administered 3,4-DHPEA is absorbed in rats and humans [25,26] and has shown antioxidant activity both in vitro [27,28] and in vivo [25,29]. The ester of 3,4-DHPEA with elenoic acid (3,4-DHPEA-EA or oleuropein) is also a potent antioxidant active in the micromolar range, and both oleuropein and hydroxytyrosol have been proven to be more potent

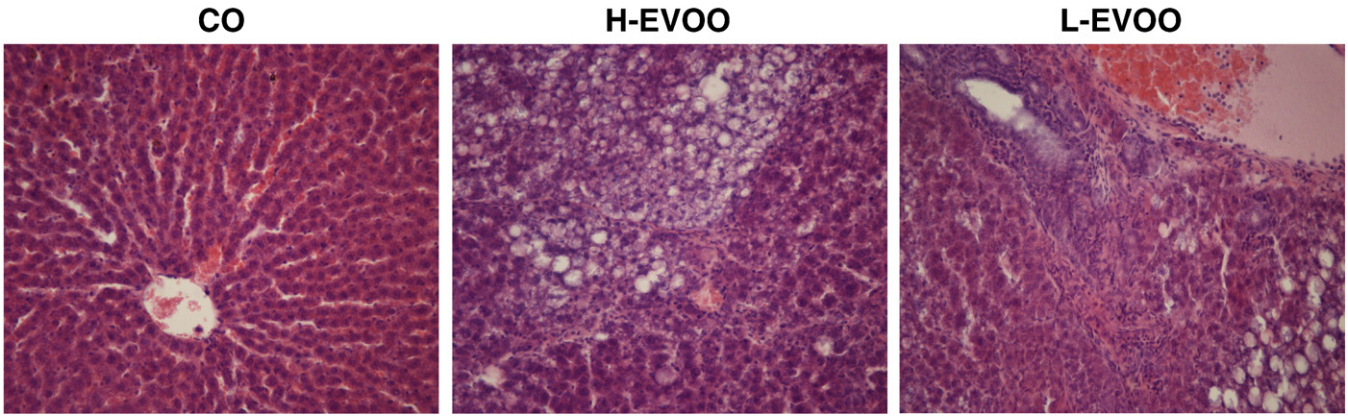

Fig. 3. Examples of hematoxylin and eosin-stained sections of livers from each group.

antioxidants than vitamin E [30]. All these compounds were present in high amounts in the olive oil selected for this study (H-EVOO), whereas they were very low in the control olive oil (L-EVOO). However, it is important to notice that, due to the procedure used in the present work for L-EVOO preparation, these two oils were identical for all the other major and minor constituents.

It is well known that during aging, a series of metabolic dysfunctions take place, leading to altered lipid and glucose metabolism, insulin resistance and organ damage. In our experiments, at the end of their life, all the animals were severely overweight (about 700 g) as compared to age-matched rats fed a lower fat diet (average body weight 550 g [31]). The animals fed corn oil had significantly lower cholesterol levels than the animals treated with either type of olive oil. Also, liver steatosis was more pronounced in the olive oil-treated animals, as indicated by the increase in organ weight and triglyceride content. An increase in the hepatic synthesis and production of triglycerides is frequently associated with insulin resistance and obesity in humans [32]. The olive oil-fed animals also showed insulin resistance in adipose tissue, at variance with those fed corn oil, and a tendency towards increased insulin plasma levels. Although we did not measure systemic insulin resistance, it has been clearly shown in a recent paper on aging Wistar rats that changes in local insulin resistance in white adipose tissue follow the same pattern of systemic insulin resistance upon either aging or dietary restriction [33]. These differences between corn oil and olive oil treatment are conceivably due to the different fatty acid compositions. Corn oil is rich in polyunsaturated ω-6 linoleic acid, which is present in low amounts in olive oil. Ω-6 fatty acids have shown a cholesterol-lowering effect in clinical studies [34], reduce insulin resistance, and their intake has been inversely related to the risk of Type 2 diabetes [35]. Corn oil also contains significant amounts of the monounsaturated oleic acid, the main fatty acid in olive oil. However, the lipid-lowering action of monounsaturated fatty acids is controversial [36,37].

One beneficial effect showed by olive oil as compared to corn oil in our experiments was the lack of increase in blood pressure at the end of the treatment. A controlling effect of olive oil on systolic but not on diastolic blood pressure has also been shown in a short-term human intervention study [38].

Regarding the antioxidant intake, since the AIN standard diet provides 0.4 mg vitamin E per rat per day, vitamin E daily intake was raised to about 0.9 mg in olive oil-treated animals due to the vitamin E content of the two oils. Extra-virgin olive oil also provided a significant amount of phenolic antioxidants (about 2 mg per rat per day total phenolic compounds, of which 3,4-DHPEA and 3,4-DHPEA-EA represented about 30%) to the diet. At the end of the treatment, the main effects of H-EVOO as compared to the other two oils devoid of phenols in the blood were a reduction in DNA oxidative damage in circulating leukocytes and plasma TBARS. In the liver, H-EVOO treatment brought about a marked increase in SOD activity accompanied by a decrease in XO activity, without detectable effects on DNA damage and lipid peroxidation. There was a tendency towards higher survival in the H-EVOO group until Week 40 of

Table 4
Effects of the experimental diets on plasma/serum parameters

	CO	H-EVOO	L-EVOO
glucose (g/L)	0.98±0.1	1.02±0.1	1.1±0.1
creatinine (mg/dl)	0.5±0.04	0.5±0.10	0.4±0.05
triglycerides (mg/dl)	134±19	199±33	150±32
cholesterol (mg/dl)	93.2±5.4	141.1±18.8 *	152.1±11.2 **
total bilirubin (mg/dl)	0.06±0.01	0.06±0.01	0.05±0.01
CPK (U/L)	7642±942	7201±1992	6810±1623
LDH (U/L)	2229±186	1930±225	1750±186
alkaline phosphatase (U/L)	138.9±11.4	192.3±49.0	121.4±19.4
amylase (U/L)	1641±115	2247±196 *	2130±456
insulin (ng/ml)	1.35±0.19	1.96±0.32	1.56±0.29

CO, corn oil; H-EVOO, extra-virgin olive oil rich in natural antioxidants; L-EVOO, extra-virgin olive oil poor in natural antioxidants.
Values are expressed as mean±S.E.M. (n=6 for insulin, n=11–13 for the other parameters, nonfasted animals). CPK, creatine phosphokinase; LDH, lactate dehydrogenase.
* P<.05 vs. CO.
** P<.01 vs. CO.

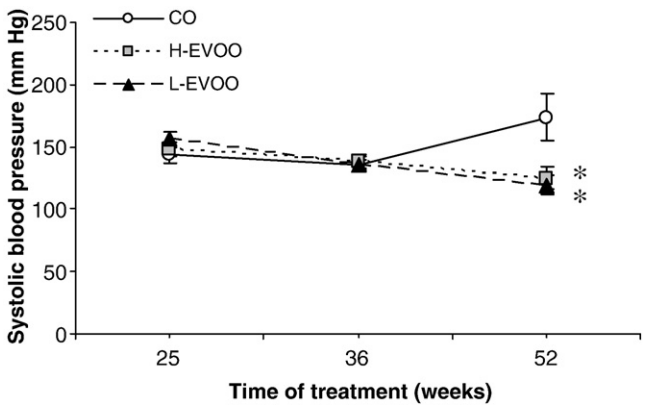

Fig. 4. Systolic blood pressure values during rat treatment. Systolic blood pressure was measured at treatment week 25, 36, 52 and values are expressed as mean±S.E.M. (10 rats per group). *P<.05 vs. CO.

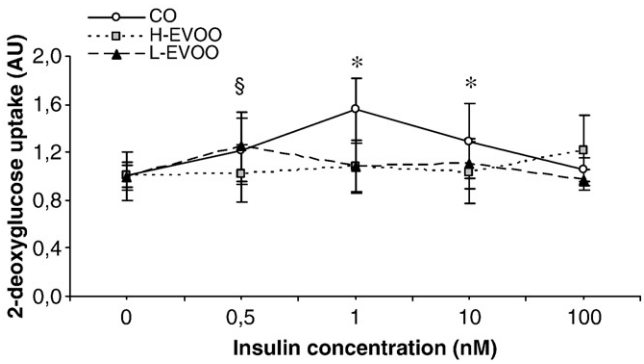

Fig. 5. Insulin-stimulated glucose uptake by epididymal adipocytes. 2-Deoxyglucose uptake was measured from epididymal fat in seven rats for each group. Uptake levels are expressed in arbitrary units (AU) (mean±S.E.M.). *P<.01, CO vs. basal. §P<.05, CO vs. basal.

treatment, possibly indicating a beneficial effect of olive oil rich in phenolic antioxidants on the general health status during aging. In agreement with these observations, a tendency towards reduction of some age-related pathologies such as hypophyseal tumors and ulcerative dermatitis was detected upon H-EVOO treatment. However, metabolic dysfunctions such as liver steatosis, dislipidemia, insulin resistance in adipose tissue, and serum amylase (an index of pancreatic damage), which were brought about by the olive oil treatment, were not modified by the presence in the oil of high amounts of phenols.

Compared to other studies where natural antioxidants have shown protective effects in aging, the daily doses used in the present work (about 4 mg/kg total phenolic compounds) were rather low, but they are the maximal that can be attained by administering natural oils and not adding phenolic antioxidants as supplements. Baur et al. [14] have reported a protective effect of chronically administered resveratrol at a dose of 22.4 mg/kg in mice kept on a high-fat diet during aging: the treated animals lived longer and showed signs of a slower aging process compared to the controls. The calorie restriction-mimetic effect of resveratrol, leading to improvement of a number of metabolic parameters, has been suggested to be associated more to its sirtuin-stimulating action rather than to its antioxidant properties. Thus, it is possible that other biological activities might be more relevant than the antioxidant effect for protection from the deleterious effects of high-fat diets.

Quiles et al. [31] have investigated the effects of lifelong olive oil feeding in rats, and found increased antioxidant capacity, reduced DNA damage and improved plasma lipid profile as compared to sunflower oil, with no change in maximal lifespan. The employed dietary concentration of olive oil in the study of Quiles was 8%, about one third of that used in the present work. It is possible that under our experimental conditions the long term damage induced by the high-fat regimen overwhelmed the protective effect of olive oil phenols. In human trials with olive oils of different phenol content, various beneficial effects have been reported in association with the phenolic intake, such as reduction in DNA oxidative damage, lipoperoxidation and other heart disease risk factors [39–42]. However, the amount of olive oil consumed daily in controlled trials is usually comprised between 25 and 50 ml per subject per day, i.e., 0.35–0.70 ml/kg body weight, whereas the amount consumed by the animals in the present work was about 2.5 ml per rat per day, corresponding to 3.5–5 ml/kg body weight. Thus, the negative effects due to the high fat intake in the animals are not present in the human trials, conceivably making it possible to better disclose both antioxidant and other beneficial effects that, in the present, work remain more elusive.

Although many epidemiological studies have associated the Mediterranean diet with longevity [43], the role of olive oil is not clear. For example, a high consumption of olive oil is frequently associated with high consumption of raw vegetables, and it is not easy to discriminate which factor is more relevant for longevity [44].

In summary, the main goal of the present work was to test the ability of food naturally containing high amounts of antioxidants to protect from the proaging effects of a long-term high-calorie diet. The doses of antioxidants used in this work had to be lower than those employed by other laboratories using pure compounds supplementing the diet. In our experimental conditions, the prolonged olive oil intake led to obesity, liver lipid degeneration and insulin resistance; these effects appeared to overwhelm any possible beneficial effect of olive oil phenols. In fact, no difference in overall survival was found in the animals treated with the phenol-rich oil compared to the other groups. Notwithstanding this, we detected higher survival at treatment Week 40 and a beneficial effect on some age-related pathologies in association with the antioxidant content of the olive oil. Furthermore, olive oil was protective of the age-related increase in systolic blood pressure. Concomitantly, some antioxidant effects were observed in blood and liver with the olive oil rich in phenols, although this did not correlate with the severity of liver steatosis. In conclusion, these data suggest that improving the quality of olive oil in terms of antioxidant content can have some beneficial effects, but it is not sufficient by itself to counteract the detrimental effects of a high-calorie diet containing large amounts of fat. Future experiments will elucidate whether the protective effects of olive oil phenols on age-related dysfunctions would be more evident using a lower-calorie and lipid intake regimen.

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