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ORIGINAL CONTRIBUTION

No effects of olive oils with different phenolic content compared to corn oil on 1,2-dimethylhydrazine-induced colon carcinogenesis in rats

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Abstract *Background* Some epidemiological and experimental studies suggest that olive oil, despite its elevated caloric content, may have protective activity against colon cancer, partially due to its phenolic content. However, little experimental evidence exists to support this claim in vivo. *Aim of the study* To test the effect of olive oils with different phenolic content in a well-characterized model of colon carcinogenesis, comparing them with corn oil (CO). *Methods* F344 rats were fed AIN-76 based diets for the entire experimental period; the diets contained 23% (w/w) of lipids from three different sources: extra-virgin olive oil rich in phenolic compounds (EV), rectified olive oil (ROO) with the same fatty acid composition but devoid of phenolic compounds and CO as a control diet. One week later, rats were induced with 1,2-dimethylhydrazine (DMH) (150 mg/kg b.w. × 2 times) to measure preneoplastic lesions (aberrant crypt foci (ACF) and mucin depleted foci (MDF)) and tumours in the intestine. *Results* Thirteen weeks after DMH, the numbers of ACF and MDF were similar in the different groups (ACF/colon were 344.9 ± 27.0, 288.6 ± 28.5 and 289.8 ± 21.4 in CO, EV and ROO groups, respectively, means ± SE; MDF/colon were 8.83 ± 1.2, 8.41 ± 1.5 and 8.75 ± 1.6 in CO, EV and ROO groups, respectively, means ± SE). Thirty-two weeks after DMH, the incidence of tumours (rats with tumours/rats in the group) did not differ among the different groups (20/21, 18/19 and 20/20 in the CO, EV, and ROO groups, respectively). Similarly, the number of tumours/ rat in the colorectum (both adenomas and cancers) was not different in the three different groups (2.33 ± 0.26, 2.42 ± 0.41 and 2.25 ± 0.40 in CO, EV and ROO groups, respectively, means ± SE). *Conclusions* Olive oil, irrespective of its phenolic content, does not affect DMH-induced colon carcinogenesis in F344 rats compared with CO.

Key words colon carcinogenesis – olive oil – corn oil – phenolic compounds – diet and cancer

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Introduction

Studies in human populations and experimental animals have suggested that high fat diets increase colon cancer risk [4, 19, 24]. Recent epidemiological data indicates that the high caloric intake associated with the high fat-diets is a very important determinant for colon carcinogenesis [25]. On the other hand experimental studies in carcinogen-induced rodents show a clear effect of fat, in quantity (diets rich in fat promoting more than low fat diets) and quality of fat [20]. For instance, it was demonstrated that while high corn oil (CO) based diets (rich in n-6 fatty acids) promote chemically induced carcinogenesis, high fat diets rich in n-3 fatty acids do not [19, 26]. The effect of other types of dietary lipids is less understood. Based on past epidemiological data on a lower colon cancer incidence in Mediterranean countries where olive oil is consumed, it has been suggested that olive oil (rich in oleic acid, an n-9 fatty acid) may have cancer protective activity, despite its caloric content [3, 16]. However, colon carcinogenesis experiments on olive oil have produced conflicting results [1, 6, 11, 17], where it has been demonstrated to reduce experimental carcinogenesis in some reports, but have no effects [6] or enhancing effects in others [17].

Recently, it has also been suggested that the protective effect of olive oil might be due to phenolic compounds such as hydroxytyrosol, tyrosol, oleuropein, particularly abundant in extra-virgin olive oil (EV) and which show anti-cancer effects in in vitro models of colon carcinogenesis [12].

Given these considerations and the availability of olive oils with the same fatty acid composition but with different phenolic content, we thought it of interest to test in a well characterized model of colon carcinogenesis, the effect of diets containing as a source of lipids extra-virgin olive oil rich in phenolic compounds (EV), or a rectified olive oil (ROO) with the same fatty acid composition of EV but devoid of phenolic compounds. A CO-based diet was used as control. Accordingly, we measured colon carcinogenesis in F344 rats induced with 1,2-dimethylhydrazine (DMH) and fed AIN-76 based diets containing 23% (w/w) lipids from three different sources: EV, ROO and CO. To test the correlation between early events of colon carcinogenesis and tumour induction, a group of animals from each dietary group was sacrificed at an earlier time point (13 weeks after DMH) and the purported preneoplastic lesions, aberrant crypt foci (ACF) [2] and mucin depleted foci (MDF) [5] were determined in the colon of these animals.

Methods

Chemicals and dietary components

DMH was purchased from Aldrich Chemical (Milan, Italy). Dietary components, except different oils were purchased from Piccioni (Gessate, Milan, Italy). Corn oil was purchased from Tampieri S.p.A (Faenza, Ravenna, Italy), while EV was purchased from Cipolloni S.p.A and Petesse S.p.A (Foligno, Perugia, Italy). The extra virgin olive oil devoid of phenolic compounds (ROO) was obtained as follows: the EV oil was homogenized for 1 min with water (1:1 v:v), and the oil separated by centrifugation (Wesfalia separator), repeating this procedure six times. The oil was then filtered with a cellulose acetate membrane.

Extraction and HPLC analysis of phenolic compounds of EV and ROO

The phenol extraction from EV and ROO was performed as reported before [14]. The HPLC analysis was conducted as reported by Selvaggini et al. [22] 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 DAD (diode array detector), and a FLD (fluorescence detector). The analysis of the oil extract was performed using Spherisorb ODS-1 250 × 4.6 mm C18 columns with a particle size of 5 μm (Phase Separation Ltd., 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. The total running time was 73 min, and the gradient changed as follows: 95% A/5% B for 2 min, 75%A/ 25% B in 8 min, 60% A/40% B in 10 min, 50% A/ 50% B in 16 min, 0% A/100% B in 14 min, maintained for 10 min. Then, initial conditions were reset and equilibration was reached in 13 min; the total running time was 73 min.

Fatty acid analysis

The fatty acids in the oils were measured with the European Official Method (UE 1989/2003 modifying the ECC 2568/91) [7].

Animal treatment

We used 4- to 5-week old male F344 rats (Nossan, Correzzana, Milan, Italy). The animals were housed in plastic cages with wire tops and maintained at a

temperature of 22°C, with a 12:12-h light-dark cycle, according to the European Union Regulations on the Care and Use of Laboratory Animals [8]. The experimental protocol was approved by the Commission for Animal Experimentation of the Ministry of Health, Rome, Italy. After their arrival from the supplier, animals (n = 97) were quarantined for a few days, during which they were fed a standard lab chow. Rats were then randomly allocated to the following experimental groups (Table 1): CO group (n = 33) was fed a high-fat diet containing 230 g/kg CO w/w as previously described [9]. This diet is based on the AIN76 diet since similar diets have been used as a reference in previous experiments on the effect of lipids (notably CO) on colon carcinogenesis [19, 20]. The EV (n = 32) was fed a high-fat diet with the same composition as the CO group except for the fat which in this case was 230 g/kg w/w of EV. The refined olive oil group (ROO) (n = 32) was fed a high-fat diet containing 230 g/kg of ROO. The composition of phenolic compounds in olive oils is given in Table 2. Table 3 shows the fatty acid composition of the different oils. Diets were prepared every 2 weeks, divided into aliquots and frozen at −20°C. Diets were fed to rats every 2 days.

Table 1 Composition of the experimental diets used in the study

Components (g/100 g diet)	CO Group	EV Group	ROO Group
Oils			
Corn oil	23		
Extra-virgin olive oil	–	23	
Rectified olive oil	–		23
Sucrose	32	32	32
Maize starch	10	10	10
Caseine	23	23	23
Cellulose	6	6	6
AIN76 Mineral mix	4	4	4
AIN76 Vitamin Mix	1.2	1.2	1.2
Coline	0.2	0.2	0.2
DL-methionine	0.3	0.3	0.3

Table 2 Phenolic compound composition of the olive oils used in the experiments (mg/kg)

Compounds	EV	ROO
3,4-DHPEA	15.0 ± 0.5	ND
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 ± 0.003
(+)-Pinoresinol	32.3 ± 0.1	2.0 ± 0.02
3,4-DHPEA-EA	214 ± 2.1	2.9 ± 0.03

The phenolic content is the mean value of three independent determinations ± standard deviations
3,4-DHPEA 3,4-dihydroxyphenyl-ethanol or hydroxytyrosol, *pHPEA* p-hydroxyphenyl-ethanol or tyrosol, *3,4-DHPEA-EDA* dialdehydic form of elenoic acid linked to hydroxytyrosol, *pHPEA-EDA* dialdehydic form of elenoic acid linked to tyrosol, *3,4-DHPEA-EA* oleuropein aglycon, *ND* not detectable

Table 3 Fatty acid composition (%) of the oils used in the experiments

	CO	EV	ROO
Saturated			
Myristic acid	ND	0.01 ± 0.001	0.01 ± 0.001
Palmitic acid	10.3 ± 1.1	12.8 ± 1.3	12.8 ± 1.3
Margaric acid	ND	0.04 ± 0.0	0.04 ± 0.00
Stearic acid	2.1 ± 0.21	1.9 ± 0.2	1.9 ± 0.2
Arachidic acid	0.7 ± 0.03	0.3 ± 0.03	0.3 ± 0.03
Behenic acid	ND	0.1 ± 0.01	0.1 ± 0.01
Lignoceric acid	ND	0.04 ± 0.004	0.04 ± 0.00
Monounsaturated			
Palmitoleic acid	ND	0.7 ± 0.1	0.7 ± 0.1
Oleic acid	27.2 ± 2.6	77.0 ± 7.7	77.0 ± 6.7
Eptadecenoic acid	ND	0.09 ± 0.009	0.09 ± 0.01
Eicosenoic acid	ND	0.3 ± 0.03	0.3 ± 0.03
Poyunsaturated			
Linoleic acid	59.4 ± 5.11	6.2 ± 0.6	6.2 ± 0.6
Linolenic acid	0.25 ± 0.03	0.6 ± 0.1	0.6 ± 0.1

Values are means of three independent determinations ± standard deviations.
nd: not detectable

One week after beginning feeding of the experimental diets, rats were administered DMH (150 mg/kg × 2 times s.c, 1 week apart). Thirteen weeks after the first DMH injection, rats from each dietary group (n = 12) were sacrificed by CO_2 inhalation to determine ACF and MDF as previously described [2, 5]. Thirty-two weeks after the first DMH injection, tumours were determined in the remaining rats as previously described [9].

Histopathological evaluation of the tumours

At sacrifice, all organs were macroscopically examined for the presence of tumours or other pathological lesions. Tissues with abnormal morphology were fixed in 10% buffered formalin and embedded in paraffin blocks. Histological sections stained with haematoxylin and eosin were used to confirm the presence and type of tumours by histopathological examination, which was performed by a pathologist unaware of the experimental codes. Suspected macroscopic lesions were measured with callipers. Tumours were evaluated on the basis of the histotype, grading and pattern of growth; adenomas were classified as tubular, tubulovillous and villous according to Morson et al. [15].

Statistical evaluation of the data

Data obtained from individual rats in the different experimental groups were summarised for quantitative continuous responses by calculating group means and standard deviations. Comparisons among the different groups were analysed with one-way ANOVA (P level fixed at 0.05, two-sided).

Results

At the beginning of the experiment rats were randomly allocated into the three dietary groups; the mean weight of the rats at this time was 113.2 g ± 0.9 (mean ± SE; $n = 97$). At the first sacrifice (13 weeks after the first injection of DMH) no statistically significant differences in body weights were found among the dietary groups (mean weights (g): 317 ± 10.1, 333 ± 7.9, 325.3 ± 8.6 in the CO, EV and ROO groups respectively, means ± SE). Similarly, no differences were found at the end of the experiment (32 weeks after DMH) when animals were sacrificed to harvest tumours (mean weights (g): 449.8 ± 4.8, 462,7 ± 9.9, 448.4 ± 10.8 in the CO, EV and ROO groups respectively, means ± SE).

Determination of the preneoplastic lesions ACF and MDF

Thirteen weeks after the first injection of DMH, rats were sacrificed to enumerate the preneoplastic lesions ACF and MDF. The results of ACF determination (Fig. 1a) showed that the rats fed the CO diet had a slightly higher number of ACF/colon than the two groups fed olive oil, however this effect did not attain statistical significance ($P = 0.22$ with ANOVA analysis). The multiplicity of ACF, i.e the number of aberrant crypts (AC) forming each focus was similar among the three groups (AC/ACF were 3.7 ± 0.1, 3.5 ± 0.1, 3.6 ± 0.1 in the CO, EV and ROO groups respectively, means ± SE).

After ACF determination the colons were stained to highlight mucin secretion as described [5] to determine MDF. The results of this analysis showed no difference among the three groups in the number of MDF/colon (Fig. 1b). Similarly, the multiplicity of MDF was not different among the three groups (crypts/MDF were 7.9 ± 1.3, 8.7 ± 0.9, 8.8 ± 0.9 in the CO, EV and ROO groups respectively, means ± SE).

Determination of tumours

Tumour induction was evaluated in 61 rats fed the three different diets (21, 20 and 20 in CO, EV and ROO groups). One rat in the EV group died before the end of the experiment (24 weeks after the first DMH injection). No colonic tumours were found in this animal that was excluded from the number evaluated. All rats were killed 32 weeks after the first DMH injection.

The majority of tumours were found in the colon and rectum. However, we found some tumours also in the small intestine and in the ear. These latter types of tumours were squamous papillomas. All the tumours found in the colon, rectum and small intestine were classified as adenomas or cancers. All adenomas were tubular. The majority of cancers were adenocarcinomas, but we also found three mucinous adenocarcinomas (one in the CO and two in the ROO groups); given the small number of samples in this category of cancers, these malignant tumours were grouped together with the adenocarcinomas and all classified as "cancers".

The incidence of tumours (rats with tumours/rats in the group) was similar among the different groups (20/21, 18/19 and 20/20 in the CO, EV, and ROO groups, respectively). Similarly, the number of tumours/ rat in the colorectum (both adenomas and cancers) in the three different groups was not differ-

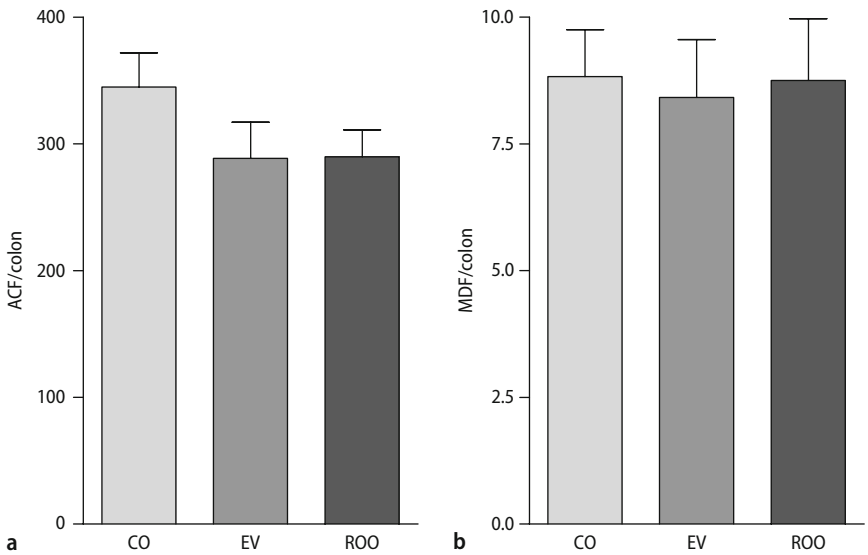

Fig. 1 **a** ACF/colon in the three different dietary groups. **b** MDF/colon in the three different groups. Values are means + SE ($n = 12$ in each dietary group)

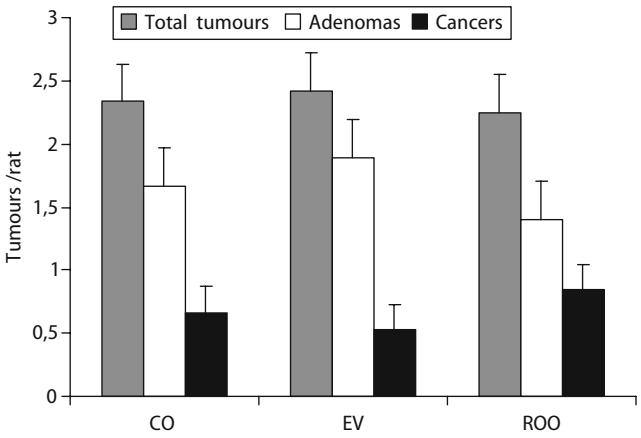

Fig. 2 Multiplicity of colorectal tumours in the three different groups. The different *bars* for each dietary group represent total tumours (sum of both adenomas and cancers), adenomas, cancers. Values are means + SE. (n = 21, 19 and 20 in CO, EV and ROO groups, respectively)

ent (Fig. 2). There were no differences in the number of tumours (both adenomas and cancers) in the small intestine (number of tumours /rat was 0.14 ± 0.08, 0.31 ± 0.08, 0.35 ± 0.18 in the CO, EV and ROO groups respectively, means ± SE). The degree of dysplasia of the adenomas as well as the grading of cancers were similar in the three different groups (data not shown).

Discussion

We tested whether olive oils with different phenolic content affect colon carcinogenesis induced in rats by DMH. Since we were also interested in studying the effect of olive oil *per se*, besides feeding a group with EV rich in phenolic compounds, we also fed animals with ROO devoid of these compounds. We then compared the groups fed olive oils with those fed a CO-based diet. The results demonstrate that these dietary variations do not affect colon carcinogenesis induced by DMH.

Previous experiments on the effect of olive oil on colon carcinogenesis have produced different results. Reddy and Maeura [20] showed that, unlike CO, olive oil fed at 23% w/w in the diet (the same level we used in this experiment) does not promote colon carcinogenesis in female rats induced with 40 mg/kg AOM. More recently, Bartoli et al. [1] report that 5% olive oil in the diet significantly reduces AOM-induced ACF but does not affect the incidence of adenocarcinomas, when compared to safflower oil (rich in n-6 fatty acid). A similar protective effect of olive oil on ACF induction was reported by Schwartz et al. [21] in rats fed 4 or 10% (w/w) olive oil, while Fujise et al. [11], show that 10% olive oil reduces incidence of cancer when compared to the same level of CO or beef tallow, in a small-scale carcinogenesis experiment. On the other hand, it has been reported that olive oil (10% in the diet) does not affect ACF induction [6], while others report that olive oil enhances colon carcinogenesis [17]. These conflicting results might be explained, at least in part, with the different protocols of administration of the carcinogen and to the type and level of fat used in the control diet, therefore, it is difficult to draw a firm conclusion on the effect of olive oil on colon carcinogenesis.

Our results, obtained in a large group of animals, clearly show that both EV and ROO do not affect DMH-induced colon carcinogenesis induced by DMH when compared with a CO-based diet containing the same level of CO. In fact, we found a similar incidence, multiplicity and differentiation of tumours in all three dietary groups. Moreover, the evaluation of two preneoplastic lesions (ACF and MDF) carried out in rats fed the same level of oil shows that olive oils, do not affect the early phases of carcinogenesis. This result, while it demonstrates a correlation between early and late events of carcinogenesis, reinforces the finding that the three different oils, at least at the level used in this experiment, have no effect on the long-term carcinogenesis experiment.

The null effect we observed might be explained by the high caloric intake produced by the relatively high amount of oil that we used (23% w/w). Calorie-rich diets are associated with high risk of colon carcinogenesis in experimental and epidemiological studies [25]. It has also been suggested that diets with high caloric and lipid content are a risk factor for colon cancer since the dyslipidemia and insulin resistance that they cause may promote colon carcinogenesis [23]. Therefore it is possible that the high caloric lipid-intake of the diets used may have overwhelmed the hypothetically protective effect of olive oil. To this purpose, it is interesting to note that the link between a low-incidence of colon cancer in certain Mediterranean countries and olive oil consumption, is challenged by recent epidemiological studies showing that Italy and Spain have an incidence similar to the mean of all European Countries [10]. Furthermore, the incidence of colorectal cancer, at least in Italy, has actually increased in recent years [13, 18].

It is also interesting to note that the multiplicity of colonic tumours in the study in which olive oil did not promote colon carcinogenesis [20], was quite low (0.46 tumours/rat), a phenomenon which might have favoured a protective action of olive oil. In the present experiment the multiplicity of colonic tumours was higher (2.4 tumours/rat), but in previous experiments with a tumour yield similar to the present one [9], we have demonstrated chemopreventive effects of dietary components.

In conclusion, EV, when compared with CO and irrespective of its phenolic content, does not affect DMH-induced colon carcinogenesis in F344 rats, at least in the experimental conditions used in this study. Thus our data do not support a protective role of olive oil against colon carcinogenesis.

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