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ORIGINAL CONTRIBUTION

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Nutritional-pharmacological combinations

A novel approach to reducing colon cancer incidence

Summary *Background* Recent studies have suggested that n-9 fatty acids in olive oil prevent colon carcinogenesis while n-6 PUFA seems to activate this process. *Aims* To evaluate the effects of nutritional-pharmacological combinations made up of olive or soy oil-based diets and the drug sulindac, on colon cancer incidence in a chemically induced (1,2-dimethylhydrazine, DMH) rat cancer model. *Methods* Male rats were assigned to two different dietary regimes based on a standard murine defined diet (AIN-76A) containing either a low (4 %) or high (15 %) concentration of olive or soy oil. Some groups also received sulindac in their food (80 mg/kg food) starting from the ninth week following the first DMH or vehicle administration. *Results* Oleic and linoleic acid reached higher levels in plasma and liver lipids when rats were fed high concentrations of olive or soy oil, respectively. Rats fed a low or high soy oil-based diet showed no significant difference in the number of aberrant crypt foci (ACF) in proximal or distal colon specimens. In contrast, rats fed a higher olive oil-based diet developed a significantly lower number of ACF than rats fed a low concentration of olive oil. Addition of sulindac reduced the number of ACF in rats fed the 4 %, but not the 15 %, soy oil diet. In contrast, the effect of sulindac was significant when combined with both the low and high concentrations of olive oil. High soy oil-based diet or DMH treatment upregulated colon expression of Bcl-2, but not that of cyclooxygenase-2 (COX-2). In contrast, olive oil dose-dependently downregulated the expression of both Bcl-2 and COX-2 in colonic mucosa and also abrogated the upregulation of Bcl-2 by DMH. Olive oil/sulindac combinations were effective in downregulating colonic mucosa Bcl-2 expression (with the 4 % oil diet) and COX-2 expression (with the 15 % oil diet). These effects were not observed in rats fed the soy oil/sulindac combinations. Caspase-3 activity in colonic mucosa was unaffected by soy oil or soy oil/sulindac combinations. The addition of olive oil, on the other hand, significantly enhanced colonic caspase-3 activity. *Conclusions* Diets containing high levels of olive oil exert a significant protective effect from tumor development that is additive with the inhibitory effect of sulindac. These inhibitory effects are mediated by regulating the expression and activity of key proteins involved in prostaglandin-biosynthesis and apoptosis-induction pathways. It may be concluded that appropriate dietary-pharmacological combination can improve anti-tumor efficacy over either dietary or pharmacological intervention alone.

Key words colon cancer – olive oil – sulindac – apoptosis

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Abbreviations

ACF	aberrant crypt foci
Cox-2	cyclooxygenase-2
DMH	1,2–1,2-dimethylhydrazine
NSAID	non-steroidal anti-inflammatory drug
PUFA	polyunsaturated fatty acid

Introduction

Environmental factors, such as diet, are believed to be significant in the etiology of a number of epithelioid cancer cases, notably colon carcinomas. In many cases, interactions between environmental and genetic elements cause a series of somatic mutations, leading to malignant transformation [1, 2]. Epidemiological data suggest that the amount and composition of dietary fat can have a significant impact on colon tumor development [3–6]. Cancer development is characterized by specific cellular transformations involving changes in proliferation rates, inactivation of tumor-suppressor genes and inhibition of apoptosis [7, 8]. Regulation of the apoptotic process involves a number of apoptosis-regulating molecules such as caspases, poly(ADP-ribose) polymerase and others [9]. Some proteins, such as Bcl-2, are considered to have anti-apoptotic activity and inhibit cell death [9].

Several studies have demonstrated that high-fat diets increase the incidence of primary colonic tumors [10], suggesting that this detrimental effect is due to the caloric consumption rather than the fatty acid composition of the diet. Nonetheless, at least one study does not support this proposition, suggesting instead that the type of fat and its unique composition are of greater importance than overall fat intake [11].

Olive oil, a major dietary source of oil in Mediterranean and African populations, has been shown to provide a relative degree of protection against colon cancer in carcinogen-induced colon cancer [12]. Olive oil is unique in its fatty acid make-up and is rich in oleic acid, a monounsaturated fatty acid (18:1). Studies indicate that by increasing oleic acid content in the diet, cancer incidence is decreased with little apparent relationship to the quantity consumed [13]. Possible cancer-prevention mechanisms mediating the oleic acid effect include elevated oxidative stress, variations in hormone levels and in the rate of apoptosis, and impact on gene expression [14–17]. The ω-6 fatty acid arachidonic acid (C20:4, ω-6) is obtained mostly from meat, or synthesized de novo from linoleic acid (C18:2, ω-6), and is a precursor of eicosanoids (prostaglandins, leukotrienes, lypoxines, thromboxans, prostacyclins). Prostaglandins are actively released during inflammation and carcinogenesis. The possible mechanisms for the pro-tumorigenic effects of ω-6 fatty acids include elevated peroxidation of structural lipids, enhanced eicosanoids (PGE2)-mediated effects, alterations in the rate of apoptosis or regulation of tumor-associated gene expression [18].

There are two cyclooxygenase (COX)[4] isoenzymes: COX-1 and COX-2. COX-1 is a constitutive enzyme expressed in the stomach, colon, kidney and blood platelets. COX-2 is an early gene response inducible enzyme, primarily upregulated during an inflammatory response in monocytic cells and fibroblasts and in many transformed cells, including colon cancer cells [19, 20]. Inhibitors of COX-2 have been demonstrated to induce apoptosis in colon cancer cells [21, 22]. Data from human trials also suggest that non-steroidal anti-inflammatory drugs (NSAID)[4], such as sulindac, can reduce the size and number of polyps in individuals with familial adenomatous polyposis [23].

Results in recent years have shown that none of the cancer therapy regimens or drugs targeted to interfere with one of the steps in the multistage carcinogenesis process are sufficiently effective to yield sustained tumor arrest when administered alone. This is due to the extraordinary ability of transformed cells to mount unique cellular mechanisms to neutralize the tumor-suppressive effect of the drug or treatment. Nutrients, which have the capacity to prevent or modify the course of development of diseases or pathological conditions, are generally much less potent than the corresponding drugs that are developed to treat such conditions. Consequently, the overall clinical efficacy of a single nutrient is limited by its individual efficacy and by the amount of food containing this ingredient that can be safely consumed. Nevertheless, because nutrients may act via unique mechanisms distinctly different from those of a specific drug, the use of dietary-pharmacological combinations may prove to be more anti-tumorigenic than either drug or nutrient treatment alone. Such an approach has the potential to yield novel dietary/drug combinations that can provide additive or even synergistic protection against cancer progression and is especially relevant when the etiology of disease development has varied mechanistic routes.

The present study was aimed at evaluating the effect of defined nutritional-pharmacological combinations, specifically soy or olive oil-based diets combined with the NSAID sulindac, on 1,2-dimethylhydrazine (DMH)[4]-induced colon cancer incidence in rats and on modulation of COX-2 levels and apoptotic pathways in rat colonic tissue.

Materials and methods

Materials

All biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified. AIN-76

mineral mix and casein vitamin free were purchased from ICN Biomedicals (Aurora, OH). Extra virgin olive oil and soy oil were purchased from Eger Farm Ltd (Yokneam, Israel). Vitamin mix was obtained from Kofolk (Petach-Tikva, Israel).

Animals

Male Sprague Dawley Charles-River-derived (inbred) strain rats (n = 160; 150 g each) were purchased from Harlan Laboratories Ltd (Jerusalem, Israel). The animals were randomly divided into four main groups, and were fed the experimental diets and provided water *ad libitum*. Animals were weighed weekly throughout the study. Research protocols and animal care were supervised by the Animal Welfare Committee of the Faculty of Agricultural, Food and Environmental Quality Sciences of the Hebrew University of Jerusalem.

Diets

The animals were fed one of the four experimental diets as described in Table 1 for 11 weeks and were then sacrificed, in protocols as described in Fig. 1. The diets were provided in powder form. The standard diet was prepared according to the recommended AIN-76A semi-synthetic diet and olive oil or soy oil was added according to the different concentrations tested (4 and 15 %). Diet intake was monitored daily.

Table 1 Dietary composition

Ingredients	Amount (g/kg)	
	Olive/soy oil[a] 4 %	Olive/soy oil 15 %
Casein	180	180
Sucrose	380	305
Starch	339.5	300
Oil source	40	150
Vitamin mix[b]	2.5	2.8[d]
Mineral mix[c]	35	39[d]
Methionine	3	3.4[d]
Cellulose	20	20
Choline chloride	0.67	0.67

[a] Source of oil: olive or soy oil, this table describes dietary composition of four different diets, containing soy oil or olive oil at two different concentrations
[b] Vitamin Mix AIN 76A (Kofolk Ltd., Petach-Tikva, Israel)
[c] Mineral Mix AIN 76A (ICN Biochemical, Cleveland, OH)
[d] Mineral, vitamin and methionine were adjusted to provide the same needs as animals fed lower concentration of fat. The lower fat diet contains 13 % less energy than the higher fat diets

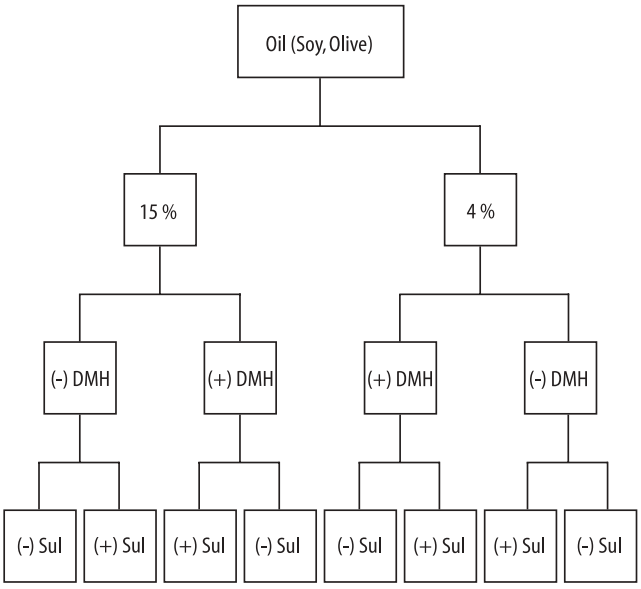

Fig. 1 Scheme depicting the protocol for 1,2-dimethylhydrazine (DMH) and sulindac (Sul) treatment. One s. c. DMH injection/week for 5 consecutive weeks was administered to the rats. Sulindac was provided in the powdered diet (four different diets containing soy or olive oil at two different concentrations) starting from the ninth week after the first DMH injection. Rats were sacrificed at week 11

1,2-Dimethylhydrazine (DMH)-induction of colon carcinogenesis

Colon cancer was induced in half of the rats (n = 80) at high efficiency by the administration of five subcutaneous DMH injections (40 mg/kg) at 1-week intervals to the different dietary groups, as previously described [24]. Eleven weeks after the first injection, rats were anesthetized with ether and blood was withdrawn from the vena cava; rats were then sacrificed by cervical dislocation.

Sulindac administration

Half of the rats in each of the dietary and/or DMH-treated rat groups (n = 10) were given sulindac daily (80 mg/kg food) in their diet, starting from the ninth week following the first administration of DMH or vehicle (see details of protocol in Fig. 1). In total, 80 rats received sulindac treatment.

Fatty acid profile in liver and serum in the different experimental groups

The distribution of the fatty acid profile in the dietary oils and serum and liver samples was determined using a GC apparatus (HP 6890, Agilent, Wilmington, DE), according to the methodology previously described by

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Friedman and Sklan [25]. Fatty acids were identified and quantified using well-defined standards.

Western blot analyses

For the immunodetection of COX-2 and Bcl-2, mucosal scrapings of colonic tissues, obtained from different experimental groups were homogenized in lysis buffer: 20 mmol/L Tris pH 7.8, 100 mmol/L NaCl, 50 mol/L NaF, 10 % glycerol, 1 % NP-40, and Complete™ protease-inhibitor cocktail at the final concentration recommended by the manufacturer (Boehringer Mannheim, Germany). The homogenates were centrifuged and supernatants were kept at –70°C for further processing. Protein concentration in the different supernatants was determined by the BCA reagent (Pierce, Rockford, IL) [25]. Protein (50-µg aliquots) was separated by 10 % SDS-PAGE and transferred to nitrocellulose filters (Schleicher and Schuell, Dassel, Germany). The filters were stained with Ponceau S to confirm equal protein loading and effective transfer. The following primary antibodies were used: rabbit polyclonal antibody to COX-2 (Upstate Biotechnology, Lake Placid, NY); and mouse monoclonal anti-Bcl-2 antibody (Zymed Laboratories, San Francisco, CA). Detection was performed with horseradish peroxidase-linked anti-rabbit or anti-mouse IgG, depending on the primary antibody [26]. Immunoreactive bands were visualized using the enhanced chemiluminescence detection reagents (SuperSignal; Pierce) as suggested by the manufacturer.

Densitometric analysis of band intensity

The total intensity of each band obtained in the western blots was detected and quantified using a PhosphorImager system (Fujix, Tokyo, Japan) and corrected for background. The values for each band represent the numbers of pixels (points) integrated by the above imaging system. Equal protein loading of each sample was verified by Ponceau S staining.

Caspase-3 proteolytic activity

Caspase-3 activity was calculated by colorimetric assay (R&D Systems, Minneapolis, MN). Colonic tissue was harvested from the different treatment groups and lysed and 200 µg-protein was tested for protease activity by the addition of a caspase-specific substrate peptide, DVED-p-nitroaniline. Caspase-3 cleavage of the peptide releases the chromophore p-nitroanilide (p-NA), which was quantitated spectrophotometrically at 405 nm. The level of caspase-3 enzymatic activity in the tissue lysate was directly proportional to the color reaction. In background reactions, no DVED-pnitroaniline substrate was added, and the values obtained were subtracted from experimental results [27–29].

Detection of aberrant crypt foci

ACF were scored in colons of rats treated with DMH, 11 weeks after the first carcinogen administration, essentially using our previously described procedure [28]. Briefly, immediately after the animals were sacrificed, colons were removed and flushed with 0.9 % NaCl solution, then opened longitudinally and fixed flat in 10 % buffered formalin. The colons were stained with methylene blue (0.1 %) for 15 min, then the mucosal side was observed at 200X magnification. Their slit-like opening increased staining, and size and pericryptal zone made ACF clearly distinguishable from normal crypts.

Statistical analyses

All values are expressed as mean ± SEM. Data were analyzed by one- or two-way ANOVA, and then differences among means were analyzed using Tukey-Kramer multiple comparison tests. Differences were considered significant at $P < 0.05$.

Results

Dietary intake

The diets were well accepted by the animals; weight gain was measured once a week and found to be similar for all experimental groups (data not shown).

Fatty acid profile analyses of soy and olive oil

The fatty acid composition of the dietary soy and olive oils provided to rats contained expected concentrations of saturated mono and polyunsaturated fatty acids typical to the respective oils: olive oil is rich in oleic acid and soy oil rich in linoleic and linolenic acid (Table 2). Oxidation status of each of the dietary oils was provided by the oil manufacturer, and showed that both oils contain similar low concentration of peroxides (Table 2).

Fatty acid profile in liver and serum in the different experimental groups

As expected, oleic and linoleic acid reached higher levels in plasma and liver lipids when rats were fed 15 % olive or soy oil, respectively, as compared to the corre-

Table 2 Fatty acid profile in soy and olive oil

Fatty acid	Experimental fats (g/100 g total fatty acid)	
	Soy oil*	Olive oil**
16:0 (Palmitic acid)	10.4	11.8
16:1 (Palmitoleic acid)	0.1	0.7
18:0 (Stearic acid)	3.0	2.7
18:1 (Oleic acid)	20.1	69.9
18:2 (Linoleic acid)	60.3	12.9
18:3 (Linolenic acid)	5.1	0.68
20:0 (Arachidic acid)	0.3	0.45
20:1 (Eicosenoic acid)	0.3	0.25

* Oxidation status: 5.2 MEQU/kg; ** Oxidation status: 4.8 MEQU/kg

sponding levels in rats fed the 4% oil diets (Table 3). DMH treatment did not affect plasma or liver fatty acid composition (data not shown).

Effect of diets and sulindac, alone or in combination, on ACF formation

Figs. 2A and 2B describe the number of ACF measured in the distal and proximal colons, respectively of DMH-treated rats fed either olive or soy oil at the two oil concentrations and additionally treated or not with sulindac. The same numbers of ACF were obtained in the proximal vs. distal areas of the colon. Rats fed high soy oil-based diets showed higher ACF numbers than rats fed lower concentrations of this diet. In contrast, a dose-dependent inhibitory effect was seen with olive oil supplementation: rats fed a high olive oil-based diet developed a significantly lower number of ACF than those fed the low concentration of olive oil ($P < 0.01$). In rats fed soy oil, sulindac was not able to significantly reduce ACF at either oil concentration. In contrast, sulindac's anti-

Table 3 Fatty acid profile in plasma and liver of rats fed with olive or soy oil diets[a]

	Plasma			
	Olive oil		Soy oil	
FA	4%	15%	4%	15%
18:1	28.8±2.6	24.4±19.2*	14.5±0.9	9.3±0.6*
18:2	9.1±0.5	10.5±0.3*	20.2±4.3	26.1±1.9*
	Liver			
	Olive oil		Soy oil	
FA	4%	15%	4%	15%
18:1	21.6±2.5	22.4±2.0	18.6±1.7	16.5±1.5*
18:2	14.6±0.7	16.6±1.0*	20.6±2.4	20.6±2.4

[a] The rats were fed for 11 weeks. Results are mean ± SD of ten rats
* $P < 0.01$

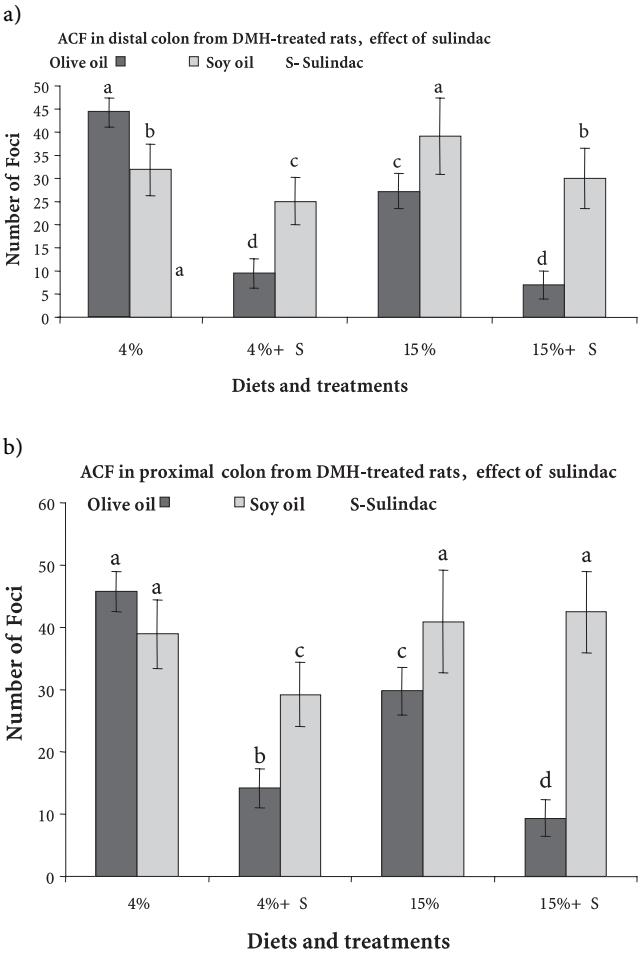

Fig. 2 Distribution of aberrant crypt foci (ACF) in the distal (**A**) and proximal (**B**) colonic regions of DMH-induced rats fed olive or soy oil with or without sulindac for 11 weeks. Data are expressed as mean ± SD of ten rats. Means without a common letter, at least $P < 0.01$

carcinogenic effect was highly significant when combined with either of the olive oil-based diets in either the distal or proximal colon ($P < 0.001$). In rats fed 15% olive oil, 80% of the ACFs consisted of five aberrant crypts (AC) or more, whereas in rats fed the same concentration of olive oil, only 8% of the ACFs consisted of five AC or more. Sulindac improved its activity when combined only with 15% olive oil, in terms of lowering the number of AC, as for ACF.

Effect of diets and sulindac, alone or in combination, on Bcl-2 expression

Feeding rats with a soy oil-based diet at high concentration induced twofold higher expression of Bcl-2 in the colonic tissue, whereas a high concentration of olive oil significantly downregulated Bcl-2 expression. In rats treated with DMH, feeding the 4% soy diet upregulated

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the expression of colonic Bcl-2 to levels similar to those obtained with the higher concentration of soy oil in the absence of DMH treatment. In contrast, olive oil at both concentrations abrogated this DMH-dependent upregulation of Bcl-2 expression (Figs. 3A, 3C). Furthermore, olive oil/sulindac, but not soy oil/sulindac, combinations were effective in downregulating the DMH-induced increased expression of Bcl-2 in colonic mucosa ($P < 0.03$) (Figs. 3B, 3C).

Effect of diets and sulindac, alone or in combination, on COX-2 expression

The high soy oil-based diet or DMH treatment did not affect COX-2 expression in colonic tissue when compared to the expression in rats fed the 4% soy oil diet (Figs. 4A, 4C). In contrast, low and high concentrations of olive oil in the diet significantly downregulated the expression of COX-2 in colonic mucosa and partially abrogated the upregulatory effect exerted by DMH (Figs. 4A, 4C). The combination of a high olive oil or soy diet with sulindac was ineffective in further downregulating COX-2 expression (Figs. 4B, 4C).

Effect of diets and sulindac, alone or in combination, on caspase-3 activity

Caspase-3 activity in colon mucosa homogenates harvested from rats fed the different experimental diets is shown in Fig. 5A. Colonic caspase-3 activity was unaffected by the soy oil diet or by the DMH treatment. In contrast, the 15% olive oil diet significantly enhanced colonic caspase-3 activity; this increase was not reversed by DMH treatment. Soy oil/sulindac combinations did not affect caspase-3 activity in the colonic mucosa (Fig. 5B). Olive oil/sulindac combinations result in significantly more caspase-3 activity in colonic mucosal preparations at only the lower olive oil dose, in animals not treated with DMH, but not in all other treatments and olive oil doses.

Discussion

Dietary fat is commonly associated with cancer risk and a positive correlation exists between dietary fat intake and increased risk for colon cancer [30]. Animal studies have shown that the colon tumor-promoting effect of a dietary fat depends not only on its amount, but also on its fatty acid composition [31–33]. By having a relative excess intake of ω-6 polyunsaturated fatty acids (PUFA)4, COX-2 enzymes are continually activated and produce excessive amounts of prostaglandins. The nutritional perspective is, therefore, to center on COX-2 as one of the main targets for modulation by nutritional intervention in order to suppress prostaglandin produc-

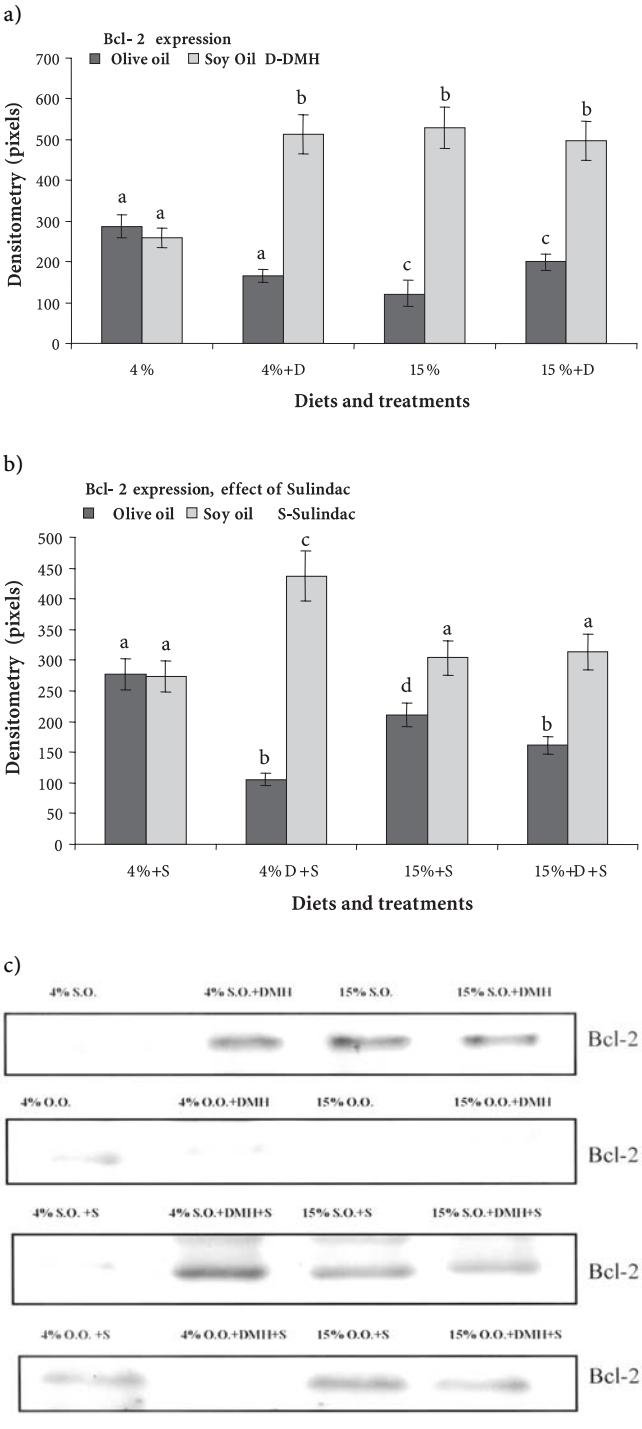

Fig. 3 Bcl-2 expression in the colon tissue of control and 1,2-dimethylhydrazine (DMH)-induced rats fed olive oil-based or soy oil-based diets and not treated (**A**) or treated with (**B**) sulindac. Results are mean ± SD of ten rats. Means without a common letter, at least $P < 0.01$. (**C**) Representative western blots demonstrating Bcl-2 expression in colonic mucosa of rats submitted to the different treatments and dietary regimes (*S. O.* soy oil; *O. O.* olive oil; *S* sulindac)

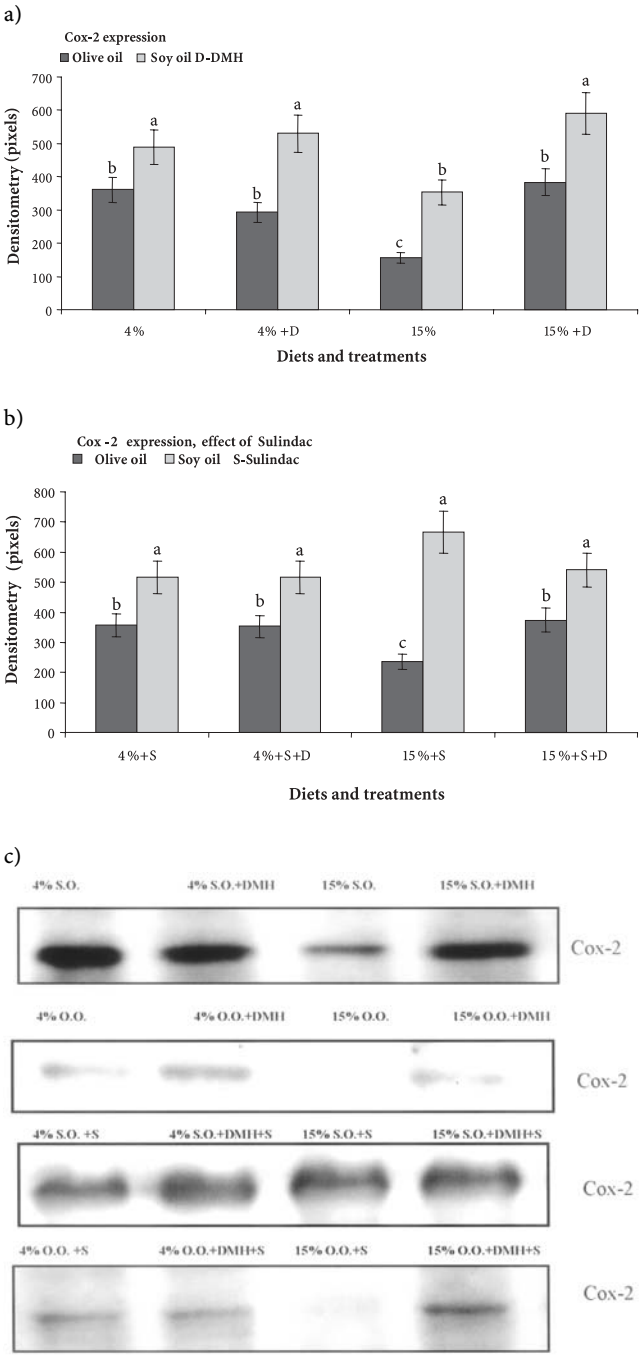

Fig. 4 COX-2 expression in the colon tissue of control and 1,2-dimethylhydrazine (DMH)-induced rats fed olive oil-based or soy oil-based diets and not treated (**A**) or treated with (**B**) sulindac. Results are mean ± SD of ten rats. Means without a common letter, at least $P < 0.01$. (**C**) Representative western blots demonstrating COX-2 expression in colonic mucosa of rats submitted to the different treatments and dietary regimes (*S. O.* soy oil; *O. O.* olive oil; *S* sulindac)

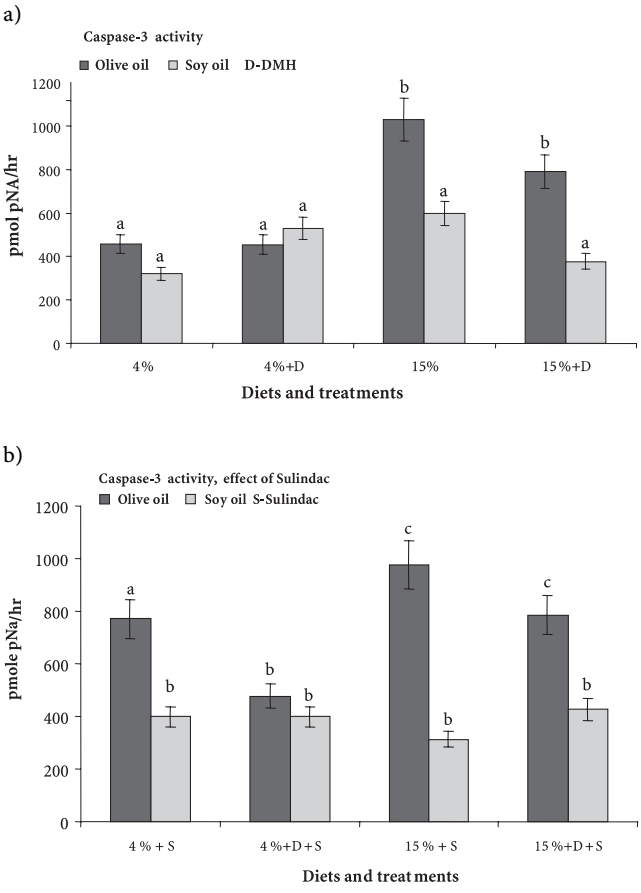

Fig. 5 Caspase-3 activity in the colon tissue of control and 1,2-dimethylhydrazine (DMH)-induced rats fed olive oil-based or soy oil-based diets without (A) or with (B) sulindac. Results are mean ± SD of ten rats. Means without a common letter, at least $P < 0.02$ (*pNa* = p-nitroanilide)

tion. One fat source documented to have a protective effect on carcinogenesis is olive oil [12, 34, 35], presumably due to its high content of oleic acid and low content of ω-6 PUFA.

The focus of the present study was to analyze the validity of nutrient/drug combinations in an animal model of colorectal cancer. The chosen nutrients consisted of diets based on different concentrations of olive oil compared to soy oil. The chosen drug was sulindac, belonging to the NSAID family. Since both the dietary oils [12, 34] and sulindac have been shown to affect cellular prostanoid formation [21], we analyzed how the nutritional regimes in the presence and absence of the drug affect COX-2 expression, and then relate these findings to protein/enzyme activities associated with apoptosis-related events.

ACF have been reported to represent pre-neoplastic lesions of colon cancer in both rodents [27] and humans [36, 37]. The number of ACF has been shown to directly correlate with the number of final tumors produced [28]. We, therefore, used this number as a bio-indicator to evaluate the anti-carcinogenic potency of the dietary-pharmacological protocols being used. Olive oil given alone exerted a cancer protective effect, which was dose-dependent on its concentration in the diet. Soy oil pro-

vided in the diet was equally ineffective at reducing precarcinogenesis at both concentrations. Combinations with sulindac were effective only when administered with olive oil. At both olive oil concentrations, sulindac significantly reduced the number of ACF. Combinations of sulindac with soy oil were equally ineffective at both low and high concentrations of the dietary fat.

Sulindac was administered 8 weeks after the first DMH administration, allowing us to assess the interaction between the different dietary regimens and the drug treatment when the drug is given in a chemotherapeutic, rather than chemopreventive, mode. The inhibitory effect of the drug on the formation of ACF was significantly increased only in the olive oil-fed rats, with maximal effect seen at the higher olive oil dose. These findings indicate that sulindac and olive oil act additively in their anticancer activities. From a mechanistic point of view, our results demonstrated that the olive oil-enriched diet significantly and dose-dependently decreases COX-2 expression in colonic tissues and concurrently attenuates the expression of the anti-apoptotic protein Bcl-2, while upregulating the activity of caspase-3. In contrast, ω-6-rich soy oil was shown to exert the exact opposite effect, i. e. the soy oil-enriched diet significantly and dose-dependently upregulated Bcl-2 expression in colonic tissues and even added to the upregulatory effect of DMH with respect to Bcl-2 expression. No anti-apoptotic-associated effect was recorded for soy oil-based diets.

In addition to oleic acid, olive oil contains a combination of antioxidants consisting of simple phenols, secoiridoids, lignans, flavonoids and squalene [38]. Some of these components have been previously shown to exert cancer-preventive effects [39]. Hence, the possibility that other components in olive oil besides oleic acid can induce an anticarcinogenic effect cannot be excluded.

In summary, the data presented here provide evidence that diets containing olive oil reduce colonic precarcinogenic events in rats, whereas soy oil-based diets upregulate these pathways. Sulindac was shown to exert an additive-synergistic effect with olive oil, thereby increasing the inhibitory effect compared to treatment with either the dietary or the pharmacological paradigm alone. This combined treatment approach may be suitable for the prevention of other types of cancers.

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