Effect of olive oil on early and late events of colon carcinogenesis in rats: modulation of arachidonic acid metabolism and local prostaglandin E_2 synthesis

R Bartolí, F Fernández-Bañares, E Navarro, E Castellà, J Mañé, M Alvarez, C Pastor, E Cabré, M A Gassull

Abstract

***Background*—Animal model studies have shown that the colon tumour promoting effect of dietary fat depends not only on the amount but on its fatty acid composition. With respect to this, the effect of n9 fatty acids, present in olive oil, on colon carcinogenesis has been scarcely investigated.**

***Aims*—To assess the effect of an n9 fat diet on precancer events, carcinoma development, and changes in mucosal fatty acid composition and prostaglandin (PG)E_2 formation in male Sprague-Dawley rats with azoxymethane induced colon cancer.**

***Methods*—Rats were divided into three groups to receive isocaloric diets (5% of the energy as fat) rich in n9, n3, or n6 fat, and were administered azoxymethane subcutaneously once a week for 11 weeks at a dose rate of 7.4 mg/kg body weight. Vehicle treated groups received an equal volume of normal saline. Groups of animals were colectomised at weeks 12 and 19 after the first dose of azoxymethane or saline. Mucosal fatty acids were assessed at 12 and 19 weeks. Aberrant crypt foci and the in vivo intracolonic release of PGE_2 were assessed at week 12, and tumour formation at week 19.**

***Results*—Rats on the n6 diet were found to have colonic aberrant crypt foci and adenocarcinomas more often than those consuming either the n9 or n3 diet. There were no differences between the rats on the n9 and n3 diets. On the other hand, administration of both n9 and n3 diets was associated with a decrease in mucosal arachidonate concentrations as compared with the n6 diet. Carcinogen treatment induced an appreciable increase in PGE_2 formation in rats fed the n6 diet, but not in those fed the n3 and n9 diets.**

***Conclusions*—Dietary olive oil prevented the development of aberrant crypt foci and colon carcinomas in rats, suggesting that olive oil may have chemopreventive activity against colon carcinogenesis. These effects may be partly due to modulation of arachidonic acid metabolism and local PGE_2 synthesis.**

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Keywords: olive oil; fish oil; azoxymethane; carcinogenesis; fatty acids; prostaglandin E_2

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Dietary fat has received considerable attention as a possible risk factor in the aetiology of colon cancer. Epidemiological studies have indicated that the amount of dietary fat is related to colon cancer incidence.[1 2] Animal studies have also consistently shown a tumour promoting effect of high fat diets (20% *v* 5% dietary fat).[3] On the other hand, similar studies have shown that the colon tumour promoting effect in animals depends not only on the amount of fat but also on its fatty acid composition. High fat corn oil based diets (23.5% n6 fatty acids) favour colon carcinogenesis, particularly in its post-initiation or promotional phase,[4 5] whereas feeding high fat diets rich in fish oil (23.5% n3 fatty acids) decreases colon tumour incidence in both the initiation and post-initiation phase.[5] The effect of olive oil on colon carcinogenesis has been scarcely studied. However, in one study, high fat diets containing olive oil (23.5% n9 fatty acids) have also been reported to have low colon tumour promoting effects.[6]

Most studies evaluating the tumour promoting effect of fat in rats have used 20% (about 5% of calories) fat diets, as this fat derived energy intake would be equivalent to that of humans in Western countries. However, 4–5% of fat (about 12% of calories) is the normal recommended amount of fat for long term feeding studies in rats.[7] Macronutrient and energy distribution of the diet is very different between humans and rats. Daily energy intake in the rat is about 1672 kJ/kg body weight, whereas in humans it is about 125–146 kJ/kg body weight. Although 20% high fat diets have been very useful for investigating the tumour promoting effect of fat in rats, the 5% level seems to be more reasonable for assessing the preventive effect of a normal content fat diet. In fact, it is not well known whether or not the effects of different fatty acid composition of diets on colon carcinogenesis persist when diets with a normal amount of fat (5% dietary fat for rats) are administered. With respect to this, it has been shown that 5% fat diets rich in eicosapentaenoic acid (EPA; n3 fatty acid) or stearic acid (saturated fatty acid) have an inhibitory effect on colon carcinogenesis as compared with 5% fat diets rich in linoleic acid (n6 fatty acid).[8 9]

Abbreviations used in this paper: EPA, eicosapentaenoic acid; ACF, aberrant crypt foci; PGE_2, prostaglandin E_2; HPLC, high performance liquid chromatography; ARA:EPA ratio, arachidonic acid to EPA ratio.

To our knowledge, the preventive effect of normal containing 5% fat diets rich in olive oil on rat colonic carcinogenesis has not been studied. This may be relevant as oleic acid (the major fatty acid in olive oil) is the main fat component of the Mediterranean diet, and olive oil has been found to be protective against oxidative stress and carcinogenesis.[10 11] A recent epidemiological study performed in France suggests that a low intake of oleic acid may increase the risk of left colon cancer.[12] Furthermore, another recent study suggests that high consumption of monounsaturated fats, mostly derived from olive oil, would be associated with a significant decrease in the risk of colorectal cancer with wild type Ki-ras genotype.[13] Therefore the present study was designed to examine the effect of a 5% fat olive oil based diet, in comparison with isolipidic fish oil and safflower oil based diets, on the early and late phases of azoxymethane induced colon carcinogenesis. The effect of the different types of fat on both mucosal fatty acid composition and local prostaglandin E_2 (PGE_2) production were also assessed.

Materials and methods

RATS AND DIETS

Four week old male Sprague-Dawley rats (about 90–100 g body weight) were purchased from B&K Universal, Barcelona, Spain. The animals were housed two to a cage in wire drop-bottom cages, to minimise coprophagia and to prevent the consumption of bedding, under controlled conditions of a 16 hour/8 hour light/dark cycle, 50% humidity, and 21°C temperature. Animals were fed ad libitum. The food cups, especially adapted for powdered diets, were replenished with fresh diet every day.

The experimental diets were prepared by Scientific Hospital Supplies (SHS) International Limited, Liverpool, UK. They were unflavoured powdered diets containing the following calculated amounts (per 100 g): fat, 5 g; protein, 20 g; carbohydrate, 64 g; fibre, 8 g; vitamins, 90 mg; minerals, 2 g; trace elements, 74 mg. The energy value for the three diets was 16.1 MJ/kg. They were made up in a defatted basic diet safflower oil (n6 diet), Ropufa* '30' n3 EPA oil (n3 diet), or olive oil (n9 diet). The Ropufa* '30' n3 EPA oil was from Roche, Basel, Switzerland, and the safflower and olive oils were supplied by Alembic Products Limited, Chester, UK. Table 1 shows the fatty acid composition of the diets as measured by gas liquid chromatography (see below). The major difference between the three diets is that the n6 diet was rich in linoleic acid, the n3 diet was rich in EPA and docosahexaenoic acids, and the n9 diet was rich in oleic acid. The diets were packaged in hermetically sealed containers with nitrogen gas and stored in a refrigerator before use.

During the 15th week of the experiment (age of animals 20 weeks), food consumption was assessed in four rats selected at random from each dietary group. Each animal was housed separately. After acclimatisation for four days, the daily dietary consumption was determined over the subsequent seven days.

Table 1 Fatty acid composition of experimental diets (mean of at least four samples of powdered diet)

Fatty acid	*n6 diet (%)*	*n3 diet (%)*	*n9 diet (%)*
14:0	0.72	6.68	1.05
16:0	9.85	19.85	19.09
16:1n7	0.18	7.70	0.79
18:0	5.23	4.26	7.63
18:1n9	19.28	13.41	56.04
18:1n7	ND	3.01	0.49
18:2n6	59.12	12.11	8.46
18:3n6	0.33	0.26	0.88
18:3n3	0.50	1.02	0.60
20:4n6	1.09	1.14	ND
20:5n3	ND	16.91	ND
24:0	ND	0.87	ND
24:1n9	0.32	0.40	0.96
22:5n6	ND	0.28	ND
22:5n3	ND	1.70	ND
22:6n3	ND	8.08	ND

ND, not detected.

The study was conducted according to the *Guide for the Care and Use of Laboratory Animals*, and was approved by the research and ethics committee of our hospital.

EXPERIMENTAL DESIGN

A total of 108 rats were randomly assigned to three dietary groups of 36 animals each (n6, n3, and n9 diets). After a one week period of adaptation, each dietary group was divided into carcinogen treated (18 rats) and vehicle treated (18 rats) subgroups. Animals intended for carcinogen treatment were given azoxymethane (Sigma Aldrich, Madrid, Spain) in saline subcutaneously once a week for 11 weeks at a dose rate of 7.4 mg/kg body weight. Animals intended for vehicle treatment were given an equal volume of normal saline subcutaneously.

At week 12, in six animals treated with azoxymethane and six controls treated with saline from each dietary group the colon was surgically removed and processed for both aberrant crypt foci (ACF) count and assay of mucosal fatty acid composition as described below. The remaining animals were colectomised 19 weeks after the first azoxymethane or saline dose, and mucosal fatty acid composition and the number, size, and location of all identifiable intestinal tumours evaluated. Only one of the animals (carcinogen treated; n3 diet) died before the scheduled termination of the experiment. Colectomy was performed through a midline laparotomy under total anaesthesia achieved with 100 mg intraperitoneal thiopental. After colectomy, animals were not allowed to recover from the anaesthesia.

In vivo local release of PGE_2 was assessed in intracolonic dialysates on the day before colectomy at week 12.

ANALYSIS OF ACF

For ACF assessment, the colons were removed, opened longitudinally, and flushed with cool normal saline. Three 0.5 cm^2 sections of the left colon, taken at 2 cm intervals, were fixed flat between filter papers in 10% buffered formalin for 24 hours. Each section was then stained with 0.2% methylene blue in saline for five minutes, rinsed in saline, and placed on microscope slides with the mucosal side up. ACFs were identified from normal crypts using a light microscope at a magnification of 40 × by their

increased pericryptal zone, elliptic or circular luminal opening, and greater thickness of the epithelial lining containing one or more crypts, which were seen to stain an intense blue.[14] Crypt multiplicity was determined as the total number of aberrant crypts per rat.

MUCOSAL FATTY ACID ANALYSIS

After colonic sections for ACF analysis had been obtained, the remaining colonic mucosa was scraped off using a microscope slide, placed in cryovials, immediately flash frozen in liquid N_2, and stored at −80°C until fatty acid assay.

Fatty acid assay was performed as previously described.[15] Tissue samples were put in a 4:1 (v/v) methanol/benzene solution and shaken for about one minute in a vortex mixer. Afterwards they were homogenised by sonication in an ultrasound bath. Direct transesterification of fatty acids was immediately carried out by the procedure of Lepage and Roy.[16] The benzene extract was evaporated under a stream of nitrogen at 40°C to complete dryness. The residue was dissolved in 100 µl benzene, and a 1 µl aliquot was injected into the chromatograph. Fatty acid methyl esters were quantified by gas liquid chromatography in a Perkin-Elmer Autosystem chromatograph (Perkin-Elmer, Norwalk, Connecticut, USA) using a 30 m capillary column, 0.25 mm internal diameter, impregnated with SP 2330 as stationary phase. The fatty acid methyl esters were identified and quantified by comparison with an external standard (Sigma Chemical, St Louis, Missouri, USA). Fatty acids from $C_{16:0}$ to $C_{24:0}$ were measured, unidentified peaks accounting for <0.5%. They were expressed as molar percentage of total fatty acids present.

INTRACOLONIC DIALYSIS AND PGE_2 ASSAY

PGE_2 release into the lumen of the colon was assessed by in vivo intracolonic dialysis as previously described.[17] Rats were anaesthetised by intraperitoneal administration of 1.5 ml/kg of a solution containing 23 mg/ml ketolar, 2 mg/ml diazepam, and 0.2 mg/ml atropine, and intracolonic dialysis was performed using hydrated Visking seamless cellulose tubing (8/32; 6.3 mm diameter; 7 cm long; Medicell International, London, UK) attached by a 10 cm polyurethane cannula to an external syringe. After the entire cannula had been inserted into the distal colon, the dialysis bag was filled with 1 ml dialysis solution, consisting of 0.3% bovine serum albumin in a solution of 120 mmol/l NaCl and 30 mmol/l $KHCO_3$ adjusted to pH 7.9. One hour later, the fluid was withdrawn and immediately stored at −80°C. A one hour dialysis period was chosen as a compromise between the time needed for equilibration and the irritant effect of the dialysis bag on PGE_2 production.[18 19]

The volume of the dialysate recovered at the end of the one hour period was larger than 90%. During this period, there was no evidence of active diarrhoea, faecal staining of the dialysis bag, or changes in dialysis fluid volume, suggesting that the PGE_2 measurements reflected production from the adjacent tissue and were not affected by fluid production in other parts of the colon.[18]

Tritiated standards of PGE_2 were purchased from Amersham International (Amersham, Buckinghamshire, UK). Standards of PGE_2 for high performance liquid chromatography (HPLC) analysis were obtained from Sigma. All solvents used in extraction and analysis were HPLC grade. Extraction of prostaglandins was performed using SepPak C_{18} Plus cartridges (Waters Associates, Milford, Massachusetts, USA) by a modification of the method of Powell.[20 21] In brief, after sample addition, the cartridges were successively washed with 10 ml bidistilled water (pH 3.15) and 10 ml petroleum ether. Afterwards, prostaglandins were eluted with 5 ml methyl formate. C_{18} cartridge recoveries were measured, after vacuum evaporation of eluates to dryness and redissolution in acetonitrile, by scintillation counting of the eluates.[20] Recovery for PGE_2 was 90.1 (2.7)% (mean (SD), n = 5).

PGE_2 determination was carried out using reverse phase HPLC.[22 23] The HPLC system consisted of a Perkin-Elmer HPLC Isocratic Lc 250 pump, and a variable wavelength (190–300 nm) Perkin-Elmer Lc 290 UV spectrophotometric detector. The reverse phase HPLC column used was a Kromasil 100 C_{18} (5 µm; 150 × 4.6 mm; Teknokroma, Barcelona, Spain). The mobile phase was water/acetonitrile (67.2:32.8, v/v), adjusted to pH 2.6 with H_3PO_4; the flow rate was isocratic to 1 ml/min and the detection wavelength 196 nm. These conditions gave a fairly good separation and resolution of prostaglandins, as previously described.[22] The eluates were collected in 1 ml fractions (FRAC 100 collector; Pharmacia Biotech, Barcelona, Spain). Using this separation, PGE_2 is eluted at 12.7 minutes and prostaglandin E_3 at 8.3 minutes. A three minute fraction (from one minute before to one minute after the elution time) was collected for PGE_2 determination. A PGE_2 EIA kit (Cayman Chemical, Ann Arbor, Michigan, USA) was used to quantify the PGE_2 in the fraction.

HISTOLOGICAL ANALYSIS OF TUMOURS

Tumours larger than 1 mm in diameter which could be identified with the naked eye were excised, fixed in 10% formalin, embedded in paraffin wax, and processed individually. The specimens were cut into 5 µm thick sections, and stained with haematoxylin and eosin for histological evaluation. All the slides were coded and examined by a pathologist who was unaware of the experimental group from which the specimens had been taken. The maximum size of each tumour was measured and the tumours were classified as adenomas or adenocarcinomas. The latter were graded by the degree of differentiation (well, moderate, or poor). Tumour incidence (% animals with tumours) and tumour multiplicity (number of tumours/animal) were recorded.

STATISTICAL ANALYSIS

Results are expressed as mean (SEM) or as proportions. χ^2 statistics were used to compare

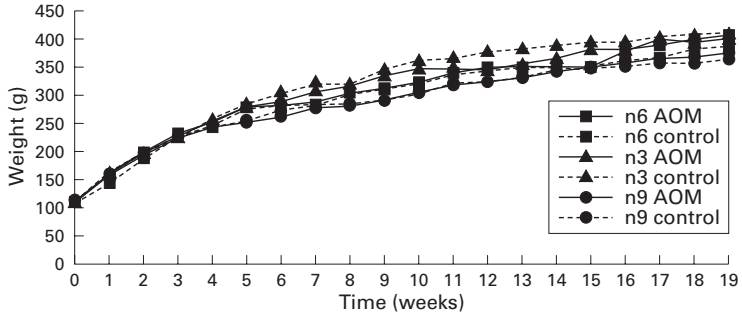

Figure 1 Mean body weights of animals over the course of the experiment.

qualitative variables. Significant differences between groups for quantitative parametric variables were evaluated using one way analysis of variance. Bonferroni test was used to assess where the differences occurred. Kruskall Wallis one way analysis of variance by rank and Mann Whitney U test were used for non-parametric variables. Statistical analysis was performed using SPSS for Windows 6.0 (SPSS Inc, Chicago, Illinois, USA).

Results

DIETARY INTAKE AND WEIGHT GAIN

There were no differences in the mean food intake either between azoxymethane and vehicle treated animals or among dietary groups. Figure 1 shows the weight of the animals over the course of the experiment. Rats in every dietary group gained weight at comparable rates, no matter what the treatment was (azoxymethane or saline), except for vehicle treated rats on the n3 diet, which gained significantly more weight than the other vehicle treated groups ($p = 0.01$ in weeks 12 and 14).

FATTY ACID ANALYSIS

Incorporation of dietary fatty acids into the colonic mucosa at week 12 was similar in saline and azoxymethane treated rats (tables 2, 3, and 4). As expected, rats fed the n9 diet had the highest mucosal concentrations of oleic acid, those fed the n3 diet had the highest concentrations of n3 long chain polyunsaturated fatty acids, and feeding the n6 diet resulted in the highest concentrations of linoleic acid in colonic mucosa.

At week 12, there were significant differences in the mucosal arachidonic acid to eicosapentaenoic acid (ARA:EPA) ratio among diets (saline and azoxymethane treated rats: $p<0.0005$; n6 *v* n9 and n3; n9 *v* n3). On the other hand, there were no differences in the mucosal fatty acid profile between azoxymethane and saline treated rats, except for animals fed the n6 diet. In these rats, carcinogen administration was associated with a significant

Table 2 Mucosal fatty acid profile (%) in rats fed the n9 diet at weeks 12 and 19 after the first dose of carcinogen or vehicle

	Week 12			*Week 19*		
n9 diet	*Control*	*AOM*	*p Value*	*Control*	*AOM*	*p Value*
SFAs	34.6 (0.45)	33.6 (0.24)	0.11	31.1 (1.7)	33.6 (1.01)	0.25
MUFAs	46.9 (1.92)	46.6 (0.83)	0.85	56.6 (5.7)	52.1 (2.9)	0.52
$C_{18:1}$n9	35.1 (1.62)	36.03 (0.78)	0.62	45.7 (5.7)	41.4 (3.1)	0.39
n3 PUFAs	1.22 (0.20)	1.28 (0.14)	0.63	0.59 (0.15)*	1.99 (0.45)	0.09
$C_{18:3}$n3	0.08 (0.02)	0.06 (0.01)	0.87	0.07 (0.01)	1.25 (0.34)†	0.055
$C_{20:5}$n3	0.15 (0.02)	0.16 (0.02)	0.94	0.09 (0.03)	0.11 (0.03)	0.62
$C_{22:6}$n3	0.80 (0.09)	0.98 (0.11)	0.25	0.41 (0.10)*	0.53 (0.12)†	0.39
n6 PUFAs	17.2 (1.88)	18.5 (0.74)	0.87	11.7 (3.8)	12.2 (2.2)†	0.91
$C_{18:2}$n6	4.15 (0.30)	4.77 (0.25)	0.33	3.41 (0.79)	3.47 (0.39)††	0.94
$C_{20:3}$n6	1.68 (0.27)	1.65 (0.09)	0.91	0.80 (0.36)	0.79 (0.19)††	0.97
$C_{20:4}$n6	9.90 (1.25)	10.36 (0.42)	0.87	6.4 (2.5)	5.7 (1.37)††	0.66
ARA:EPA	63.6 (5.5)	69.4 (7.44)	0.55	77.1 (6.7)	56.1 (12.3)	0.20

Values are expressed as mean (SEM).
*p<0.05 *v* controls at week 12.
†p<0.05 and ††p<0.01 *v* AOM at week 12.
AOM, azoxymethane; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; ARA:EPA, arachidonic acid to eicosapentanoic acid ratio.

Table 3 Mucosal fatty acid profile (%) in rats fed the n3 diet at weeks 12 and 19 after the first dose of carcinogen or vehicle

	Week 12			*Week 19*		
n3 diet	*Controls*	*AOM*	*p Value*	*Controls*	*AOM*	*p Value*
SFAs	38.3 (1.27)	36.9 (1.21)	0.48	31.9 (1.93)*	32.7 (3.2)	0.25
MUFAs	35.9 (3.10)	37.8 (1.43)	0.60	31.8 (1.04)	32.2 (1.84)†	0.86
$C_{18:1}$n9	23.9 (2.20)	25.5 (1.19)	0.57	19.5 (3.03)	16.8 (3.9)†	0.58
n3 PUFAs	10.8 (2.20)	9.8 (1.07)	0.67	15.4 (1.07)	13.6 (1.6)	0.36
$C_{18:3}$n3	0.12 (0.02)	0.13 (0.01)	0.95	0.88 (0.73)	1.64 (0.92)	0.53
$C_{20:5}$n3	5.76 (1.27)	5.46 (0.74)	0.84	8.3 (0.95)	5.4 (0.61)	0.024
$C_{22:6}$n3	3.48 (0.76)	2.88 (0.30)	0.48	4.17 (0.26)	3.9 (0.26)†	0.50
n6 PUFAs	14.9 (1.81)	15.4 (1.51)	0.83	20.8 (2.27)	21.5 (3.35)	0.87
$C_{18:2}$n6	7.08 (0.46)	7.22 (0.31)	0.82	7.65 (0.69)	7.01 (0.81)	0.57
$C_{20:3}$n6	1.22 (0.23)	1.48 (0.24)	0.46	1.83 (0.55)	2.32 (0.70)	0.59
$C_{20:4}$n6	5.33 (1.00)	5.39 (0.85)	0.96	6.92 (0.56)	5.2 (0.59)	0.053
ARA:EPA	1.00 (0.10)	0.97 (0.06)	0.78	0.86 (0.07)	0.96 (0.06)	0.63

Values are expressed as mean (SEM).
*p<0.05 *v* controls at week 12.
†p<0.05 *v* AOM at week 12.
AOM, azoxymethane; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; ARA:EPA, arachidonic acid to eicosapentanoic acid ratio.

Table 4 Mucosal fatty acid profile (%) in rats fed the n6 diet at weeks 12 and 19 after the first dose of carcinogen or vehicle

	Week 12			Week 19		
n6 diet	*Controls*	*AOM*	*p Value*	*Controls*	*AOM*	*p Value*
SFAs	34.3 (0.30)	35.8 (0.49)	0.022	33.7 (0.50)	35.5 (0.44)	0.025
MUFAs	23.9 (0.22)	22.8 (0.62)	0.12	25.8 (1.07)	24.3 (0.74)	0.52
$C_{18:1}$n9	16.9 (0.21)	15.8 (0.46)	0.06	19.8 (0.87)*	18.4 (0.77)†	0.42
n3 PUFAs	0.63 (0.03)	0.62 (0.04)	0.93	0.81 (0.07)*	0.65 (0.03)	0.08
$C_{18:3}$n3	0.08 (0.01)	0.08 (0.01)	0.82	0.10 (0.01)	0.07 (0.01)	0.08
$C_{20:5}$n3	0.11 (0.003)	0.12 (0.02)	0.37	0.11 (0.01)	0.11 (0.01)	0.81
$C_{22:6}$n3	0.39 (0.02)	0.37 (0.02)	0.49	0.46 (0.06)	0.38 (0.02)	0.11
n6 PUFAs	41.1 (0.44)	40.6 (0.91)	0.61	39.6 (0.63)	39.5 (0.45)	0.87
$C_{18:2}$n6	16.1 (0.48)	16.7 (1.20)	0.64	19.5 (1.06)*	18.02 (1.25)	0.20
$C_{20:3}$n6	2.42 (0.15)	2.84 (0.15)	0.08	1.70 (0.20)*	1.88 (0.12)††	0.42
$C_{20:4}$n6	19.9 (0.56)	18.4 (0.26)	0.051	15.8 (1.00)**	16.9 (1.19)	0.26
ARA:EPA	189.2 (8.8)	161.6 (23.2)	0.26	146.4 (11.4) *	152.2 (9.1)	0.99

Values are expressed as mean (SEM).
*p<0.05 and **p<0.01 *v* controls at week 12.
†p<0.05 and ††p<0.01 *v* AOM at week 12.
AOM, azoxymethane; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; ARA:EPA, arachidonic acid to eicosapentanoic acid ratio.

increase in the molar percentage of saturated fatty acids.

At week 19, the differences in the ARA:EPA ratio between the diets were similar to those at week 12 (saline and azoxymethane treated rats: p<0.0005; n6 *v* n9 and n3, and n9 *v* n3). On the other hand, there were only slight differences in the mucosal fatty acid profile between azoxymethane and saline treated rats, with a significant decrease in the concentrations of EPA in the n3 diet group, and an increase in saturated fatty acid concentrations in animals fed the n6 diet.

Animals fed the n9 diet showed a decrease in all n6 polyunsaturated fatty acids at week 19 as compared with week 12, particularly azoxymethane treated rats. Mucosal arachidonate concentrations decreased significantly in this dietary group, reaching values similar to those of animals fed the n3 diet, and significantly lower than those in the n6 diet group (azoxymethane treated rats: p = 0.003; n6 *v* n3 and n9). In addition, rats fed the n9 diet showed a significant decrease in docosahexaenoic acid ($C_{22:6}$n3) and a significant increase in α-linolenic acid ($C_{18:3}$n3) at week 19. On the other hand, rats in the n3 diet group showed a significant decrease in oleic acid and an increase in docosahexaenoic acid concentrations at week 19 as compared with week 12, especially azoxymethane treated rats. In addition, azoxymethane treated rats in the n6 diet group showed a slight but significant increase in oleic acid concentrations and a decrease in dihomogammalinoleic acid ($C_{20:3}$n6) concentrations at week 19.

ASSAY OF PGE_2

Figure 2 gives intracolonic PGE_2 concentrations at week 12. Azoxymethane treated rats fed n6 fat released significantly higher amounts of PGE_2 into the colonic lumen than both the other azoxymethane treated groups and the vehicle treated rats. In contrast, rats on the n3 diet treated with the carcinogen showed a slight but significant decrease in the release of PGE_2, and rats in the n9 diet group did not show any change compared with vehicle treated rats. On the other hand, PGE_2 release in vehicle treated

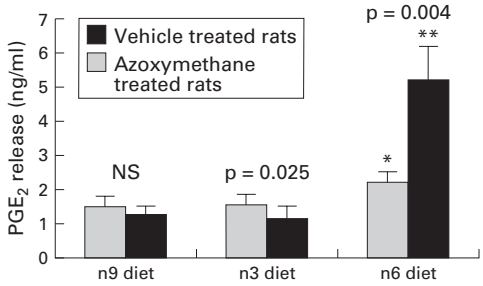

*Figure 2 Effect of dietary fat on intracolonic prostaglandin E_2 (PGE_2) release 12 weeks after the first dose of carcinogen or vehicle. Results are mean (SEM); n = 4 to 6 per group. *p = 0.02 compared with results for the n9 diet; **p = 0.002 compared with results for the n3 and n9 diets.*

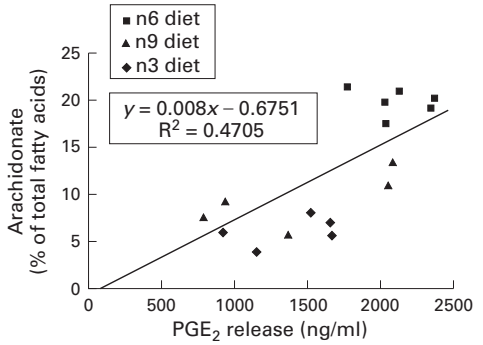

Figure 3 Significant correlation between mucosal arachidonate concentrations and dialysate prostaglandin E_2 (PGE_2) release in vehicle treated rats.

n9 diet fed rats was significantly lower than in the n6 diet group.

A significant correlation between mucosal arachidonate concentrations and intracolonic PGE_2 release was observed in both vehicle treated (r = 0.686; p = 0.002) and carcinogen treated rats (r = 0.889; p<0.0005) (figs 3 and 4). Whereas in vehicle treated rats this correlation was linear, in azoxymethane treated animals it was logarithmic because of the pronounced increase in PGE_2 concentrations in the rats fed the n6 diet.

ACF FORMATION

Figure 5 shows the number of ACF and crypt multiplicity. Animals administered saline showed no evidence of ACF formation in the

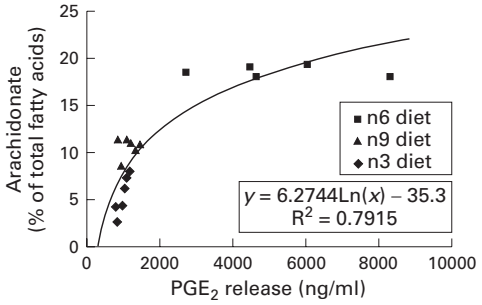

Figure 4 Significant correlation between mucosal arachidonate concentrations and dialysate prostaglandin E_2 (PGE_2) release in carcinogen treated rats.

colon, whereas all azoxymethane treated rats developed ACF. In animals fed the n9 and n3 diets, azoxymethane induced significantly less ACF per rat than those fed the n6 diet (overall p = 0.0062; n9 and n3 diets *v* n6 diet). This was mainly due to a decrease in foci with one or two aberrant crypts in the n9 diet group, and in foci with four crypts in the n3 diet group. However, the total number of aberrant crypts per rat (crypt multiplicity) was also significantly lower in both the n9 and n3 diet groups than in the n6 diet group (overall p = 0.0044).

TUMOUR FORMATION

Animals treated with saline showed no evidence of tumour formation at either 12 or 19 weeks. Likewise, azoxymethane treated rats showed no evidence of tumours at week 12. However, 19 weeks after the first injection of azoxymethane, colonic adenocarcinomas developed in seven of 12 (58%) rats of the n9 diet group, in five of 11 (45%) rats fed the n3 diet, and in 10 of 12 (83%) rats of the n6 diet group (p = 0.15). Neither benign adenomas nor metastatic invasion of the colonic tumours to the liver, peritoneum, or regional lymph nodes were observed. The number of malignant colonic tumours per rat was 0.8 (0.2), 0.7 (0.3), and 2.5 (0.8) for the n9, n3, and n6 diet groups respectively (p = 0.03; n9 and n3 *v* n6 diet). Tumour localisation was different in the different dietary groups. Thus animals on the n3 and n6 diets had a predominance of colon tumours in the distal half of the colon (seven of eight tumours and 28 of 30 tumours respectively) as compared with those on the n9 diet (five of 10 tumours) (p = 0.006; n9 *v* n6 groups). Mean tumour size was not significantly different among the groups. However, animals fed the n9 and n3 diets showed a lower percentage of poorly differentiated adenocarcinomas than those fed the n6 diet (table 5).

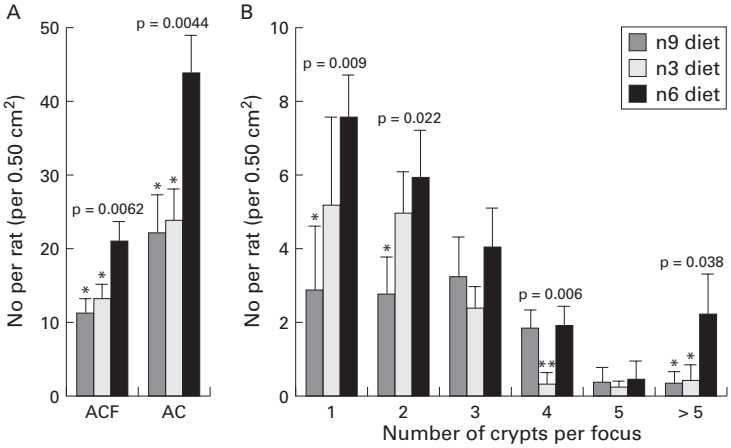

*Figure 5 (A) Comparison of total number of aberrant crypt foci (ACF) and total number of aberrant crypts (AC) per rat by dietary group (n = 6 per group); (B) comparison of the total number of ACF by number of crypts per focus and by dietary group (n = 6 per group). Results are mean (SEM) per 0.50 cm^2. *p<0.05 v n6 diet; **p<0.05 v n6 and n9 diets.*

Table 5 Histology of colon tumours in azoxymethane treated rats 19 weeks after the first dose of carcinogen

Histological parameter	*n9 diet (adenocarcinomas=10)*	*n3 diet (adenocarcinomas=8)*	*n6 diet (adenocarcinomas=30)*
Well differentiated	3 (30)	2 (25)	6 (20)
Moderately differentiated	7 (70)	4 (50)	6 (20)
Poorly differentiated	0	2 (25)	18 (60)**

Numbers in parentheses are percentage of tumour per total colon tumours with each diet.
**p = 0.01 n6 *v* n9 diet.

Discussion

The preventive or inhibitory effect of n3 fatty acids (fish oil) on experimental colon carcinogenesis has been widely evaluated.[3 5 9 24] Fish oil has also been reported to suppress rectal cell proliferation in both healthy human subjects[25] and patients with colonic adenomas.[26] In contrast, the effect of n9 fatty acids on colon carcinogenesis has been scarcely assessed. Thus, the results of this study showing that an experimental diet containing 5% olive oil has an anticarcinogenic effect similar to that of n3 fatty acids may be of relevance.

The use of precancer events to predict the effects of diets on colon carcinogenesis is of great interest because it would simplify the experimental design of animal studies. We chose to investigate alterations in the occurrence of ACF as surrogate biomarker of carcinogenic changes in the rat colon during the initiation phase of colon carcinogenesis.[27–30] ACF have been recently reported to represent preneoplastic lesions of colon cancer in both rodents[31] and humans.[32 33] However, known inhibitors of experimental colon carcinogenesis do not always prevent ACF formation.[34] This may be related to differences in both the experimental model and the carcinogenic regimen used. Thus, contrasting the effect of interventions on the premalignant biomarkers with their late effects on colon cancer development, as shown in this study, seems to be a prudent approach. In the present work, n9 and n3 diets significantly inhibited ACF formation, and both types of diet similarly inhibited the subsequent development of colonic tumours when compared with n6 fatty acids. In fact, the effects of these diets on the number of ACF paralleled the effects on tumours. Thus, using the present experimental design, the effect of dietary fatty acids on ACF numbers seems likely to be a useful predictor of tumour occurrence.

It has been suggested that ACF formation and ACF growth should be examined independently, as an intervention could presumably suppress the initial formation of single aberrant crypts and/or prevent single ACF from increasing in size.[34] In this study, the n9 and n3 diets

decreased the number of ACF similarly. However, feeding the n9 diet was mainly associated with a reduction in ACF with one or two crypts, whereas n3 diet consumption predominantly decreased ACF with four crypts. In spite of this, there were no differences in the later development of colon carcinoma between these two groups. In agreement with these findings, the size of a focus (number of aberrant crypts in a focus) was not predictive of subsequent development of tumours in a recent study.[35] The differences in ACF size in rats on the n3 and n9 diets suggest that these fatty acids act in different phases of ACF formation and growth, and thus the possibility that they may act synergistically should be explored.

Besides the incidence and multiplicity of tumours, we observed differences in the degree of differentiation of carcinomas between diets. Previous studies have produced contradictory results in relation to the effect of the n3 diet on tumour differentiation. Recently, it was shown that dietary n3 fats reduce tumour yields and improve the degree of differentiation of carcinomas at colorectal anastomosis in rats.[36] These findings contrast with a previous paper showing that, although dietary EPA reduced tumour yield, the tumours were not as well differentiated as those from animals fed n6 fat.[9] The present findings, showing a trend to a higher degree of differentiation with n3 diets as compared with n6 fat, are in agreement with the first study. However, the significant effect of n9 fat on the degree of differentiation has not been previously described. These results suggest an effect of n3 and n9 fatty acids on cell differentiation; the intrinsic mechanisms of such effects should be further investigated.

The antitumour effect of a fish oil diet has been attributed to its content of EPA rather than docosahexaenoic acid.[37] In humans, fish oil supplementation, which is associated with a doubling of the rectal mucosal EPA content, significantly decreased the rectal mucosa cell proliferative indices.[26] In addition, a stepwise reduction in tissue EPA values in the human colonic adenoma dysplasia carcinoma sequence has been described.[15] On the other hand, it has been suggested that the dietary n3 to n6 ratio is involved in determining the effects of fish oil on rectal cell proliferation in humans.[38] In that study, fish oil supplementation in subjects eating a high fat n6 diet had no antiproliferative effect, suggesting that in these subjects n3 fat supplementation was not able to decrease the mucosal ARA:EPA ratio, in spite of increasing EPA concentrations.[38] Therefore, it seems that the goal of dietary interventions in colonic carcinogenesis should be to decrease the mucosal ARA:EPA ratio instead of merely increasing mucosal EPA concentrations. On the other hand, the antitumour effect of olive oil has been ascribed, in part, to its oleic acid content, the predominant fatty acid in olive oil (about 75%). The present study shows that the n9 diet significantly decreased both mucosal arachidonate concentrations and ARA:EPA ratio as compared with the n6 diet, which may in part account for the observed beneficial effect of olive oil. In addition to oleic acid, other components of olive oil, such as squalene, and flavonoid and polyphenolic compounds may have chemopreventive activity against colon carcinogenesis. With respect to this, results of a recent study showed that 1% squalene suppressed colonic ACF formation and crypt multiplicity in a model of experimental colonic carcinogenesis.[39] Thus the antitumour effect observed with the n9 diet in the present study may also be due to its squalene content (0.8% of the olive oil used in the n9 diet).

Oleic acid is also found in the fat of beef and poultry (30–45% of the fat) and in other vegetable oils, such as corn oil (30%), soybean oil (25%), and sunflower seed oil (33%).[40] As these other fats and oils containing oleic acid generally act as promoters of chemically induced tumours in animals, it has been suggested that the oleic acid content of olive oil cannot account for its protective effect in cancer development.[40] However, these other fats and oils are rich in either saturated fat or linoleic acid, and their administration is associated with an increase in the mucosal arachidonate content, and thus with an increase in the ARA:EPA ratio.

Methods for studying the role of eicosanoids in colon carcinogenesis include various in vitro assays, such as determination of the mucosal PGE_2 concentration in tissue homogenates,[9] generation and release of PGE_2 by colonic biopsy specimens into the cell culture medium,[25] and formation of prostaglandins from [^{14}C]arachidonate through mucosal cyclooxygenase activity.[41] However, measurements of tissue concentrations may be misleading because eicosanoids are not stored, but produced in response to tissue trauma—for example, mucosal trauma resulting from biopsy sampling may itself activate membrane bound phospholipases.[42 43] Similar caveats apply to tissue culture techniques.[44] Over the last few years, in vivo intracolonic dialysis against rectal mucosa, as performed in our study, has been suggested to be a more reliable index of the balance between eicosanoid production and degradation than in vitro incubations of colon mucosa. In fact, it has been widely used to assess in vivo eicosanoid release in experimental and human colitis.[17–19 45 46] Rectal dialysate collections probably arise from production by the adjacent colon without any contribution from secretions from other parts of the intestine.[16 46] On the other hand, although with a dialysis time of only one hour our measurements may have underestimated PGE_2 concentrations, they avoided the artificial formation of PGE_2 induced by the irritant effect of the dialysis bag. Nevertheless, dialysate PGE_2 concentrations were considerably enhanced in the carcinogen treated animals fed the n6 fat diet as compared with the n3 and n9 groups, and correlated closely with mucosal arachidonate concentrations, suggesting that they are a good estimation of the mucosal production of this prostaglandin and are not derived from bacterial microflora.

As mentioned above, one possible mechanism by which n3 and n9 fats may exert their

antitumour effect may be by influencing arachidonic acid metabolism. This fatty acid is in turn the precursor of the dienoic prostaglandins, and it has been shown that PGE_2 stimulates in vitro colon cancer cell proliferation[47] and inhibits apoptosis,[48] suggesting a potential role for the arachidonate derived eicosanoids in human colon carcinogenesis. In this respect, previous studies have shown increased concentrations of PGE_2 in colon cancer tissues in both humans and rats.[41 49 50] It has been suggested that n3 fats may exert their anticarcinogenic effects by decreasing membrane arachidonic acid concentrations by competitive substitution,[37] thereby reducing the synthesis of PGE_2.[9 25 41] Results of the present study are in agreement with this hypothesis, as n3 fat suppressed the excessive production of PGE_2 that accompanied ACF formation in the n6 fat diet group. On the other hand, competitive substitution of membrane arachidonate by n9 fats was also associated with unchanging intracolonic PGE_2 release, suggesting that the antipromotional effect of n9 fats may also be mediated through inhibition of the formation of the dienoic prostaglandins. In addition to curtailing the formation of arachidonate metabolites, it has been suggested that the antipromotional action of n3 fats may be mediated by other mechanisms such as effects on oncogene expression,[51–53] apoptosis,[54–56] and intracellular signal transduction pathways.[41 57–59] Whether these mechanisms are also involved in the effects of n9 fats has to be further evaluated.

In conclusion, this study provides evidence that a 5% fat diet containing olive oil as compared with a 5% safflower oil diet prevents colonic carcinogenesis in rats, as occurs with 5% fish oil diets. This effect may be partly due to the modulation of arachidonate metabolism and local PGE_2 synthesis. Whether there is a direct inhibitory effect of olive oil and fish oil on colon carcinogenesis, or whether there is only a deleterious effect of safflower oil needs further investigation.

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Effect of olive oil on early and late events of colon carcinogenesis in rats: modulation of arachidonic acid metabolism and local prostaglandin E_2 synthesis

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