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An n-3 PUFA-rich microalgal oil diet protects to a similar extent as a fish oil-rich diet against AOM-induced colonic aberrant crypt foci in F344 rats

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ABSTRACT

The chemopreventive effects of high fat microalgal oil diet on azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) were studied in male Fischer 344 rats following 8 weeks of dietary treatment. These effects were compared to the effects of high fat fish oil and high fat corn oil diets to determine whether microalgal oil is a good alternative for fish oil regarding protection against colorectal cancer. Despite the difference in fatty acid composition and total amount of n-3 polyunsaturated fatty acids (PUFAs) between microalgal oil and fish oil, both these oils gave the same 50% reduction of AOM-induced ACF when compared to corn oil. To determine whether oxidative stress could play a role in the chemoprevention of colorectal cancer by n-3 PUFAs, feces and caecal content were examined using the TBA assay. The results showed that lipid peroxidation does occur in the gastrointestinal tract. As several lipid peroxidation products of n-3 PUFAs can induce phase II detoxifying enzymes by an EpRE-mediated pathway, the *in vivo* results suggest that this route may contribute to n-3 PUFA-mediated chemoprevention. All in all, n-3 PUFA-rich oil from microalgae is as good as fish oil regarding chemoprevention in the colon of the rat.

1. Introduction

The relationship between nutrition and cancer is complex. However, there is epidemiological, clinical, and experimental evidence that dietary fish oil, containing n-3 polyunsaturated fatty acids (PUFAs), protects against the development of colorectal, endometrial, breast, and prostate cancer (Hong et al., 2000; Narayanan et al., 2001; Stoll, 2002; Terry et al., 2002, 2003; Augustsson et al., 2003; Cheng et al., 2003; Dommels et al., 2003b). The use of PUFA-rich fish oil in foods has disadvantages due to the possible presence of environmental contaminants such as PCBs and dioxin-like compounds. The quality of the fish oil is variable and depends on fish species, seasons and origin. Furthermore, marine fish oil is a complex mixture of fatty acids with varying lengths and amounts of double bonds, that may require expensive purification before application. The demands for n-3 PUFAs are rapidly increasing, due to a rapid increase in aquaculture and applications in food and pharmacy. It is therefore expected that in the near future, the production of PUFAs from current sources will become inadequate for supplying the expanding market (Sijtsma and de Swaaf, 2004).

Alternative processes for PUFA-production are currently being exploited and further studied; one of these is the exploitation of microbial PUFA sources like microalgae; i.e. *Crypthecodinium cohnii* and *Schizochitrium* sp. (Sijtsma and de Swaaf, 2004). Apart from being a primary source of PUFAs, the fatty acids from microalgae have further advantages over fish oils, such as: the lack of unpleasant odor, the potential for better purification, and a relatively consistent spectrum of fatty acids (Pulz and Gross, 2004). Over the last few years, the commercial production of n-3 PUFA-rich microalgal oils has grown and microalgal oils are already used in 84% of US infant formulas.

Many studies conducted in rat have indicated that the incidence of chemically induced colon tumors is significantly lower in rats receiving a fish oil-enriched diet than in those receiving a saturated fat or vegetable oil-enriched diet (Minoura et al., 1988; Nelson et al., 1988; Reddy and Sugie, 1988; Deschner et al., 1990; Latham et al., 1999; Rao et al., 2001). The use of macroscopic tumors as end points has its drawbacks, because tumor development takes 5–8 months, and each tumor must be confirmed by histology. Furthermore, large groups of rats are needed for statistical analysis (Corpet and Taché, 2002).

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; HFCO, high fat corn oil; HFFO, high fat fish oil; HFMO, high fat microalgal oil; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid reactive substances.

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As an alternative method, aberrant crypt foci (ACF) can be used to study modulators of carcinogenesis. It provides a simple, relatively fast, and economical tool for the preliminary screening of potential carcinogens and chemopreventive agents, and, in the last decade, ACFs have been used as a useful biomarker for studying colon tumor modulation in rodents (Wargovich et al., 1996; Corpet and Taché, 2002). Characteristics of ACF that can be quantified include: the total number of ACF per colon, the size (area) of individual ACF, and the crypt multiplicity comprising each focus (Magnuson et al., 1993). The most common parameter measured is the number of ACF in all or part of the colon. There have been some studies that found crypt multiplicity to be a better predictor of tumor outcome than the number of ACF (Pretlow et al., 1992; Zhang et al., 1992; Magnuson et al., 1993). That is why both parameters will be used in this study. However, one has to keep in mind that in spite of articles that have proven the use of ACF as a validated method to predict carcinogens and chemopreventive agents, there are articles in literature that contradict these outcomes (Wijnands et al., 2004).

The main goal of this study was to compare microalgal oil with fish oil regarding the anticarcinogenic properties towards colorectal cancer. As far as we know, this is the first time that microalgal oil is tested for its chemopreventive character. An n-3 PUFA-rich microalgal oil was tested via an AOM-induced ACF experiment in male Fischer 344 rats. The modulating effects of a high fat microalgal oil (HFMO) diet were investigated, and compared to high fat fish oil (HFFO) and high fat corn oil (HFCO) diets. The characteristics of ACF were quantified for the total amount of ACF per colon, the crypt multiplicity per focus and whether the focus was present in the proximal or distal part of the colon. The actual fatty acid composition of the three oils was quantified and although the three oils differed in composition other than in fatty acid composition, it is assumed that these differences will not give rise to a difference in anticarcinogenic potential of the oils.

In previous studies conducted by the authors and others, it is suggested that n-3 PUFAs have to be oxidized to induce the mechanism by which n-3 PUFAs are chemopreventive (Dommels et al., 2003a; Levonen et al., 2004; Gao et al., 2006; van Beelen et al., 2006a,b). Therefore, in this study this hypothesis was tested by using the TBA assay, measuring lipid peroxidation in the feces and caecum content of the tested animals (Buege and Aust, 1978).

2. Materials and methods

2.1. Chemicals

Azoxymethane (⩾90%C – CAS: 25843-45-2), butylated hydroxytoluene (⩾99% – CAS: 128-37-0), chloroform (⩾99% – CAS: 67-66-3), methyleicosanoate (⩾98% – CAS: 95799-86-3), 1,1,3,3-tetramethoxypropane (99% – CAS: 102-52-3), 2-thiobarbituric acid (⩾98% – CAS: 504-17-6), trimethylsulphonium hydroxide (purum – CAS: 17287-03-5), and polyunsaturated fatty acid mix No. 2 (animal source) and No. 3 (menhaden source) were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA). Concentrated hydrochloric acid (37% – CAS: 7647-01-0), ethanol (⩾99.8% – CAS: 64-17-5), and trichloroacetic acid (⩾99% – CAS: 76-03-9) were obtained from Merck (Darmstadt, Germany). Phosphate buffered saline (Dulbecco 'A' tablets) was obtained from Oxoid (Harlem, The Netherlands).

2.2. Animals and housing

Thirty three-weeks-old male Fischer 344 rats were purchased from Charles River Breeding Laboratory (Someren, The Netherlands) and acclimatized for 1 week. The rats were housed in macrolon type 2 cages (450 cm^2) – two rats per cage – in an animal holding room using a 12-h light/dark cycle. Temperature and humidity were controlled at 20–24 °C and 50–60%, respectively. The animals were stratified by body weight into three dietary sub-groups of ten rats, so that mean initial body weights did not differ (mean initial bodyweight is 59.3 ± 4.5 g/rat).

2.3. Diets

The rats were given *ad libitum* access to feed and water. Feed in powder form was provided to the animals freshly each day. The experimental diets, HFMO, HFFO and HFCO, were formulated on the basis of a modification of the modified AIN-93 diet by Dommels et al. (Table 1) (Dommels et al., 2003b). The diet, as described by Dommels et al., was modified in the way that no tertiary butyl hydroquinone had been added, as this compound is known for its inducing effect on the expression of phase II and antioxidant genes (van Beelen et al., 2006a). The oils used were: (high oleic) corn oil (Remia, Den Dolder, The Netherlands), Omegapure® Refined Menhaden Oil (Omegaprotein, Houston, TX, USA), and Martek *life'sDHA*™ oil (Martek Biosciences Corporation, Columbia, MD, USA). The feed was stored in daily portions at −20 °C.

Table 1
Composition of the experimental diets.

Dietary ingredients	HFCO[a]	HFFO[a]	HFMO[a]
Casein	25.00	25.00	25.00
L-Cystine	0.37	0.37	0.37
Wheat starch	37.54	37.54	37.54
Cellulose	6.18	6.18	6.18
Choline bitartrate	0.25	0.25	0.25
AIN-93 minerals	4.32	4.32	4.32
AIN-93 vitamins	1.24	1.24	1.24
Corn oil	25.00	5.00	5.00
Fish oil	0.00	20.00	0.00
Microalgal oil	0.00	0.00	20.00
A-tocopherol acetate	0.10	0.10	0.10
Peroxide values oils used[b]	1.5	3.1	0.3

[a] Values are expressed as percent by weight.
[b] Peroxide values are expressed in mEq/kg fat.

3. Experimental procedure

Per dietary subgroup 10 animals were used. All animals were given subcutaneous injections of AOM in sterile isotonic salt solution (PBS), once weekly for 2 weeks, at a dose of 15 mg/kg body weight, after 2 weeks of experimental diet (Dommels et al., 2003b). The animals were placed in an isolator during the 2 weeks of the AOM treatment period (week 3 and 4). Fresh feces were collected at several days by gently coercing feces from the rectum. The body weight of the animals was monitored during the study.

At the end of the experiment (week 8) the animals were put under anesthesia – a combination of isofluran, O_2 and N_2O – and the colon and caecum were removed. The caecum was snap-frozen in liquid nitrogen and stored at −80 °C until further processing.

3.1. Quantification of colonic aberrant crypt foci

Colons were slit open lengthwise. After the contents of the colon were removed, the colons were spread flat between sheets of filter paper, and fixated using 10% formalin in PBS solution. After fixation the colons were stored in 70% ethanol. The colons were stained using 0.5% methylene blue in PBS for 30 s. Excess methylene blue was removed from the colons by rinsing with PBS. In a randomized, double-blind trial the number of ACF per colon, the number of aberrant crypts in each focus, and the location of each focus was determined by microscopy at a magnification of 40×.

3.2. Lipid analysis

A solution was made of 4.0 mg oil per 1.0 ml chloroform containing 1.0 mg/ml methyleicosanoate (C20:0) as an internal standard. For GC-analysis 200 μl of the solution was used, to which trimethylsulphonium hydroxide was added to produce methyl esters of the fatty acids (Butte, 1983). The fatty acid methyl esters were analyzed on a Carlo-Erba GC, equipped with a 25 m × 0.25 mm (*df* = 0.20 μm) Chrompack CP-58 CB column. The column temperature was raised from 150 °C to 250 °C with 10 °C/min and kept at 250 °C for 10 min. Samples (1.0 μl) were injected at a temperature of 275 °C. For detection the build-in flame ionization detector was set at a temperature of 280 °C. Response correction factors for flame ionization detectors were used to convert to