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In Vivo Regulation of Colonic Cell Proliferation, Differentiation, Apoptosis and P27^{Kip1} by Dietary Fish Oil and Butyrate in Rats

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Abstract

We have shown that dietary fish oil is protective against experimentally-induced colon cancer and the protective effect is enhanced by co-administration of pectin. However, the underlying mechanism(s) have not been fully elucidated. We hypothesized that fish oil with butyrate, a pectin fermentation product, protects against colon cancer initiation by decreasing cell proliferation and increasing differentiation and apoptosis through a p27^{Kip1} mediated mechanism. Rats were provided diets of corn or fish oil, with/without butyrate, and terminated 12, 24 or 48 h post azoxymethane (AOM) injection. Proliferation (Ki-67), differentiation (Dolichos Biflorus *Agglutinin*), apoptosis (TUNEL) and p27^{Kip1} (cell cycle mediator) were measured in the same cell within crypts in order to examine the coordination of cell cycle as a function of diet. DNA damage (N^7-methylguanine) was determined by quantitative immunohistochemical analysis. Dietary fish oil decreased DNA damage by 19% (P=0.001) and proliferation by 50% (P=0.003) and increased differentiation by 56% (P=0.039) compared to corn oil. When combined with butyrate, fish oil enhanced apoptosis 24 h post AOM injection compared to a corn oil/butyrate diet (P=0.039). There was an inverse relationship between crypt height and apoptosis in fish oil/butyrate group (r= −0.53, P=0.040). Corn oil/butyrate group showed a positive correlation between p27^{Kip1} expression and proliferation (r= 0.61, P=0.035). These results indicate the in vivo effect of butyrate on apoptosis and proliferation is dependent on dietary lipid source. These results demonstrate the presence of an early coordinated colonocyte response by which fish oil and butyrate protects against colon tumorigenesis.

Keywords

fish oil; butyrate; colon cancer; cell

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Introduction

There is accumulating epidemiological, clinical and experimental evidence showing that dietary modification is an important factor in the prevention of colon cancer (1). We have previously shown that, using an AOM-induced colon carcinogenesis model, a fish oil/pectin diet protects against colon cancer by lowering tumor incidence (2), and DNA damage at the initiation stage of colon carcinogenesis (3) compared to a corn oil/cellulose or corn oil/pectin diet.

Dietary fish oil, rich in n−3 polyunsaturated fatty acids (PUFA), i.e., eicosapentaenoic acid (EPA, 20:5n−3) and docosahexaenoic acid (DHA, 22:6n−3), protects against colon cancer by altering cell kinetics (i.e. proliferation, differentiation and apoptosis) (3–8). Pectin is a soluble fiber and it is fermented by colonic microflora, which produces short chain fatty acids (e.g., acetate, propionate and butyrate), CO_2, methane and other gases (9). Among the short chain fatty acids, butyrate is used as a major energy source by colonocytes (10, 11). Even though some controversies remain concerning the in vivo responses of colonocytes to butyrate exposure (9), butyrate has been shown to impact cell kinetics, e.g. cell proliferation, differentiation and apoptosis, which regulate development of colon carcinogenesis (12–16). Therefore, we hypothesized that the combination of fish oil with butyrate would protect against colon cancer initiation through an integrated pattern of changes in cell proliferation, differentiation, apoptosis and $p27^{kip1}$ protein levels. This hypothesis was further supported by a very recent study (17) showing that pescovegetarians (vegetarians who consume fish and seafood products) have a much lower risk of developing colorectal cancer, indicating potential benefit from the interaction of n−3 PUFA and dietary fiber.

To date, a number of investigators have measured proliferation and apoptosis in the same preclinical model. Even though data generated at the animal target tissue level are informative, they show only an averaged overall trend. Measuring these variables in the same cell offers the potential to determine the coordination of physiological phenomena in a cell. Furthermore, even though $p27^{Kip1}$ is a well-established inhibitor of cell proliferation, $p27^{Kip1}$ may also regulate differentiation and apoptosis (18, 19). However, the connection of $p27^{Kip1}$ with proliferation, differentiation and apoptosis is not clear. By determining these four variables in the same cell, we were able to provide an opportunity to gain greater understanding of the regulation of cell kinetics in individual cells. To our knowledge, this study is the first to determine all four of these variables in the same cell.

Materials and Methods

Animals and study design

The animal use protocol was approved by the University Animal Care Committee of Texas A&M University. Forty-eight male weanling Sprague Dawley rats (100–120g) (Harlan, Houston, TX) were individually housed and maintained in a temperature and humidity-controlled animal facility. This study was a 2×2×4 factorial design with two types of fat (corn oil or fish oil), with or without butyrate, and at four time points (0, 12, 24 or 48 h) post carcinogen injection. Rats were provided with the defined diets for 3 wk before carcinogen azoxymethane (AOM, 15 mg/kg of body weight, s.c.). Rats had free access to food and water at all times and 48h food intake was measured after 2 wk of receiving diets. Body weights were recorded weekly throughout the study.

Diets

The four defined diets (Table 1) differed in lipid source (corn oil or fish oil) and the administration of butyrate pellets. The major differences between the fatty acid compositions of the two lipid sources were significantly higher amounts of EPA (20:5, n−3) and DHA (22:6, n−3) in the fish oil compared to corn oil diet, and higher amounts of 18:2 (n −6) in the corn oil diet. Diets were prepared based on the standard AIN-76A formulation with modification of 15% fat amount, corn oil or fish oil (Vacuum-deodorized Menhaden fish oil, OmegaPure, Houston, TX) (20). Diets contained equivalent amounts of antioxidants; 26 mg α-tocopherol, 14 mg γ-tocopherol and 2 mg tertiary butylhydroquinone (TBHQ)/100g diet. Gastro-resistant slow-release butyrate pellets (S.A. Valpharma, Serravalle, Italy) designed to be primarily released in the colon (20) were supplemented (1.5 g/100 g diet) into the diets. A higher butyrate concentration in the feces was reported in rats fed with these pellets (20).

Tissue acquisition

Rats were euthanized by CO_2 asphyxiation, and the entire colon was immediately resected. After removal of the rectum, 1 cm of the distal colon was fixed in 4 % paraformaldehyde and another 1 cm was fixed in 70% ethanol. From the paraformaldehyde-fixed tissue section and serial sections were cut; one was used for colocalization of proliferation and $p27^{Kip1}$, and the other for colocalization of differentiation and apoptosis. Since ethanol fixed tissue was used for quantitative localization of N^7-methylguanine, N^7-methylguanine was not part of the colocalization protocol but was measured in a tissue section adjacent to that used for the paraformaldehyde fixed sections.

Colocalization of cell proliferation and $p27^{Kip1}$

Antigen was retrieved by microwave treatment in 0.1 M sodium citrate buffer (pH 6.0). To block non-specific background staining, tissue sections were incubated with normal rabbit serum (Jackson, West Grove, PA) in normal sheep serum (Jackson). A mixed solution of monoclonal anti-Ki-67 antibody (BD Biosciences, San Diego, CA) and rabbit anti-$p27^{Kip1}$ antibody was used as the primary antibodies. Slides were incubated with biotinylated sheep anti-mouse IgG (Jackson) and Texas-Red Streptavidin (Vector laboratory Inc., Burlingame, CA). Subsequently, slides were incubated with fluorescein-labeled goat anti-rabbit IgG (Jackson). Finally, slides were counterstained and mounted with DAPI/antifade (Vector) and were stored at 4 °C before imaging. Omission of each or both primary antibodies was used as a negative control. At least 20 crypt columns per animal were chosen for quantitative analysis.

Images of colonic crypts were visualized using a Nikon Eclipse TE300 microscope (Nikon Inc., Melville, NY, USA) equipped with an FITC filter (excitation 490–505 nm/ emission 515–545 nm) and a Texas-Red filter (excitation 560–585 nm/ emission 600–652 nm). Images were captured using a Micromax 5 MHz cooled digital CCD camera (Princeton Instruments, Trenton, NJ, USA) with a constant exposure time and 2 × 2 binning. The position of Ki-67 positive stained cell and the staining intensity of p27^{Kip1} were assessed by cell position within the crypt using a MetaMorph Imaging System (Version 4.6r3, Universal Imaging Corp., Downingtown, PA, USA). For cell proliferation, the proliferation index was calculated as 100 times the number of labeled cells per crypt column divided by the total number of cells per crypt column (21). Proliferative zone was calculated as 100 times the position of the highest labeled cell divided by the number of cells per crypt column (21). For p27^{Kip1} quantitation, nuclei on one side of a crypt column were outlined and the staining intensity was measured. Background staining intensity was subtracted from the staining intensity of the nuclei. Optimum offset and gain were determined by preanalysis of multiple darkly- and lightly-stained tissues to maximize the distribution of stain intensity so that small differences in staining were quantifiable. For accuracy and consistency purposes, once established, the settings remained constant for all images.

Colocalization of differentiation and apoptosis

Apoptosis was detected using ApopTag fluorescein in situ apoptosis kit (Intergen, Purchase, NY) and differentiation was determined using Dolichos Biflorus *Agglutinin* (DBA) glycoprotein staining. DBA has a carbohydrate specificity to α-N-actylgalactosamine (22). This carbohydrate residue is thought to increase with normal differentiation of colonic epithelial cells (23, 24).

Antigen sites in these tissue sections were retrieved by treatment in Proteinase K (5 μg/ml). Tissues were incubated with non-fat milk and then incubated with biotinylated DBA (10 μg/ml, Vector). Slides were incubated with Texas-Red conjugated streptavidin (Vector). Preincubation of 0.2 M N-acetyl-D-galactosamine (Sigma, St. Louis, MO) with DBA or omission of DBA was used as a negative control for DBA staining. TdT enzyme was applied to tissue sections and then sections were incubated in anti-digoxigenin-fluorescein in blocking solution. Omission of TdT enzyme was used as a negative control and DNAse I treated tissue section was used as a positive control for apoptosis. At least 20 crypt columns per animal were randomly chosen for quantitative analysis. The position of DAB-stained cells and apoptotic cells were recorded using a MetaMorph Image system. Images were captured using the same inverted fluorescent Nikon microscope as described above. The differentiation index and apoptosis index were calculated as previously described (24).

In vivo measurement of N^7-methylguanine DNA adducts

DNA damage was measured by quantitative immunohistochemistry using a rabbit polyclonal antibody to N^7-methylguanine (gift from Dr. Geoff Margison, Paterson Institute for Cancer Research, Manchester, UK). Tissue sections were placed in prewarmed 50 mM NaOH/40% ethanol at 55 °C to denature DNA and neutralized with 5 % acetic acid/40 % ethanol. Sections were incubated with the primary antibody followed by biotinylated goat anti-rabbit IgG. The antibody-antigen complex was visualized using the DAKO Liquid DAB (diamino-benzidine tetrahydrochloride) Substrate-Chromagen System (DAKO, Carpinteria, CA). Liver N^7-methylguanine DNA adducts in AOM-injected animals were used as a positive control. Omission of primary antibody was used as a negative control. Images of colonic crypts were visualized on a MICROSTAR IV, Reichert light microscope, captured by a digital camera (Sony DXC-970 MD, color 3CCD) and staining intensity (assessed by cell position) was analyzed using NIH Image software (NIH Image, version 1.61). Each nucleus on one side of a crypt column was outlined and the staining intensity was measured. Background staining intensity was subtracted from the staining intensity of the nuclei.

Statistical analyses

Proliferation, p27^{Kip1}, differentiation, apoptosis and DNA damage data were analyzed using three-way ANOVA to determine the effect of fat, butyrate and time. When $p < 0.05$ for the interactions, means of all diet groups were separated using Fisher's Protected Least Significant Difference (LSD) test. When $p < 0.05$ for the effects of fat, fiber or time but not for the interaction, overall means for fat, fiber or time were separated using the Fisher's LSD test. The correlations between variables were tested using Pearson's correlations, and statistical significance was assessed using Fisher's distribution, with calculations performed using PROC CORR in SAS (SAS Institute Inc. Cary, NC).

Results

There were no significant differences in food intake or body weight gain among any of the four treatment groups (data not shown).

Images of colocalization of proliferation, p27^{Kip1}, differentiation and apoptosis

Figure 1A shows a representative image of colocalization of proliferation (shown in orange) and p27 (shown in green). Proliferation was predominantly localized in the lower region of the crypt. Green fluorescence (p27^{Kip1} expression levels) in epithelial cells was quantitatively assessed in each epithelial cell.

In each serial section (Fig. 1B), differentiation (shown in red) and apoptosis (shown in green) were colocalized. Lectin DBA was primarily localized in the upper part of the crypt where the greatest numbers of differentiated cells are expected. The steady state level of apoptosis was low and frequently found in the upper portion of the crypt. In contrast, following carcinogen injection, apoptosis increased particularly in the bottom part of the colonic crypt (Fig. 1B).

Carcinogen effects on DNA damage, proliferation, p27^{Kip1}, differentiation and apoptosis

N^7-methylguanine DNA adducts, as an indicator of DNA damage, increased by12 h following AOM injection and then started to decrease by 48 h post AOM injection ($P < 0.001$, Fig. 2A). Cell proliferation decreased at 12 h after carcinogen injection and returned to basal levels at 48 h after AOM injection ($P < 0.001$, Fig. 2B). In contrast, the level of p27^{Kip1} was not changed by AOM injection (Fig. 2C). Differentiation was increased at 24 h post AOM injection ($P = 0.014$, Fig. 2D) and apoptosis was maximized at the same time point ($P < 0.001$, Fig. 2E).

Dietary fat and butyrate effects on DNA damage, proliferation, p27^{Kip1}, differentiation and apoptosis

Dietary fish oil resulted in lower DNA adduct levels compared to corn oil throughout all time points ($P = 0.001$, Fig. 3A). Even though there was no butyrate effect on cell proliferation, there was an obvious main effect of dietary lipid after carcinogen injection. Dietary fish oil decreased cell proliferation index compared to the corn oil diet (P = 0.003, Fig. 3B main graph). At 48 h post carcinogen injection, there was a compensatory increase in cell proliferation above that observed at 0 h in corn oil-fed rats (10.2% to 18.5%) but not in fish oil-fed rats (9% to 8.41%) (P = 0.010) (Fig. 3B, inset). The pattern of changes in proliferative zone over time, as a function of dietary fat type, was similar to proliferation index over time (data not shown). There was no significant effect of dietary fat or butyrate on $p27^{Kip1}$. Dietary fish oil increased differentiation compared to the corn oil diet (P = 0.039, Fig. 3C), and butyrate treatment also increased differentiation (P = 0.041) (Fig 3C, inset). There was no main effect of dietary fat or butyrate on apoptosis. When the data were analyzed using a subplot analysis for the different time points, the fish oil/butyrate diet increased the apoptotic index compared to the other groups at 24 h post carcinogen injection (P = 0.039, Fig. 3D).

Correlation among DNA adducts, proliferation, $p27^{Kip1}$, differentiation and apoptosis

After carcinogen injection, there was a decrease of proliferation and increase of apoptosis (Fig. 2B & 2E), which was associated with a decrease of crypt height (Fig. 4A). There was no significant correlation between proliferation and crypt height (data not shown). There was an inverse relationship between crypt height and apoptosis in fish oil/butyrate treated animals (coeffiecient = −0.52, P = 0.040) (Fig. 4B). However, a significant inverse relationship was not detected in corn oil/butyrate group (Fig. 4B).

As expected, DNA adduct levels were low in the saline injected rats, and there was no correlation between adduct levels and proliferation (Fig. 4C). However, in carcinogen injected animals, there was an inverse relationship between DNA adduct levels and cell proliferation (coefficient = −0.42, P = 0.010). In saline groups, there was an inverse relationship between DNA damage and apoptosis (coefficient = −0.70, P = 0.012) (Fig. 4D). In contrast, there was a positive relationship between DNA damage and apoptosis after carcinogen injection (coefficient = 0.36, P = 0.033).

To further explore the interactive effects of dietary fat and butyrate treatment on $p27^{Kip1}$, a positive relationship between $p27^{Kip1}$ level and proliferation in the corn oil/butyrate group (coefficient = 0.61, P = 0.035) was observed (Fig. 5). In contrast, there was no significant correlation between $p27^{Kip1}$ level and proliferation in fish oil/butyrate diet (Fig. 5). Interestingly, at the same expression level of $p27^{Kip1}$, the corn/butyrate diet was associated with a higher level of proliferation compared to the fish oil/butyrate diet.

Discussion

The normal colon is a dynamic tissue dependent upon an equilibrium among cell proliferation, differentiation and death (25). Colon tumors are initiated by nuclear damage leading to a disturbance of this steady-state. In this study, the relationships among cell proliferation, differentiation, apoptosis and a cell cycle mediator $p27^{Kip1}$ were determined using a colocalization technique that allowed for the determination of all four variables in the same cell. To the best of our knowledge, this study is the first work using this approach to examine regulation of cell kinetics during cancer initiation and the response to diet. The associations between DNA damage and the four variables were also assessed.

Accumulating evidence indicates that dietary fish oil protects against colon cancer (2–8). The current data also strongly indicate that dietary fish oil decreased DNA damage and cell proliferation, and increased differentiation. At 48 h post AOM injection, corn oil-fed animals had a colonocyte proliferation index that was elevated above the levels observed at 0 h (control). In comparison, fish oil-fed rats exhibited restoration of colonocyte proliferation to a level similar to those at 0 h, which may result in lower propagation of DNA damaged cells in the fish oil-fed animals. The apoptotic index was increased, but only when rats received the combination of fish oil and butyrate.

Even though it is known that butyrate modulates cell cycle kinetics, the effect of butyrate on colon cancer development is still debated (9). Results from some studies suggest that butyrate is chemopreventive by decreasing tumor growth via a reduction in cell proliferation and an increase in differentiation and apoptosis (13, 20, 26). The administration of 1% butyrate in drinking water has been reported to increase the percentage of rats with colonic tumors (27). Others report no benefit of butyrate administration with respect to aberrant crypt foci (ACF) formation when slow-release butyrate pellets were provided to rats consuming a corn oil diet (28). However, our laboratory has shown that butyrate in combination with fish oil decreased ACF formation compared to corn oil/butyrate diet (20). These studies (20, 28) in combination with data from our study suggest that whether or not butyrate is protective against colon carcinogenesis depends on the type of dietary fat consumed. Consistent with this finding, dietary fish oil decreased cell proliferation and increased differentiation compared to the corn oil diet. However, when butyrate was combined with fish oil, this diet also increased apoptosis, whereas the pro-apoptotic effect of butyrate was not present when it was combined with corn oil.

Apoptosis plays an important role in tissue homeostasis by eliminating damaged cells, suggesting its importance in cancer therapy and the prevention of carcinogenesis (29, 30). With respect to molecular mechanisms of action, mounting data confirm that the inhibition of this process may be a critical event in the development of colonic tumors (3, 24, 29, 30). Cyclooxygenase-2 (COX-2) is an enzyme that converts arachidonic acid (20:4, n−6) to prostaglandin E_2, and has been reported to be involved in colonic tumor development (31, 32). Studies have shown that prostanglandins produced by COX-2 promote colon cell proliferation and inhibit apoptosis (32). The increase of cell proliferation and resistance to apoptosis that occurs in colon cancer cell lines and in animal models of the disease was reversed by Sulindac, a COX-2 specific inhibitor (33, 34). When COX-2 is over expressed, butyrate does not induce apoptosis in rat intestinal epithelial cells (35). We and others have previously shown that n−3 enriched fish oil compared to n−6 rich corn oil reduces the level of mucosal arachidonic acid (36), COX-2 expression (37, 38) and alters the relative levels of prostaglandin E_2 and prostaglandin E_3 (39) in rat colonocytes. Thus, fish oil compared to corn oil decreases COX-2 expression and its pro-tumorigenic metabolites, thereby generating an environment in which butyrate can induce apoptosis. Further studies are needed to understand the mechanisms whereby fish oil and butyrate are able to effect changes in proliferation and apoptosis through COX and prostaglandin pathways.

The enhancement of apoptosis with fish oil feeding in combination with butyrate was also verified in experiments using ex vivo isolated epithelial cells. Cells from fish oil-fed rats incubated with butyrate induced apoptosis via alteration of mitochondrial function (decrease of mitochondrial membrane potential, cytochrome C release from mitochondria and increase of caspase 3 activity) compared to corn oil and butyrate incubated cells (40). In addition, it was reported that fish oil/pectin (butyrate generating fiber) diets decrease anti-apoptotic bcl-2 expression followed by increased apoptosis (41). The enhanced apoptosis was attributed to a reduction of bcl-2 via methylation of bcl-2 promoter region in fish oil/pectin group (42). In contrast, corn oil with butyrate treatment increased bcl-2 levels in mouse colonic cells (43). The proapoptotic effects of fish oil/pectin diet were also associated with the suppression of peroxisome proliferator-activated receptorδ (PPARδ) and PGE_2 and increase of PGE_3 (39) and modulation of microRNA and mRNA expression profiles (44).

In the present study, when butyrate was combined with a corn oil diet, there was a positive relationship between $p27^{Kip1}$ and cell proliferation. The increase in $p27^{Kip1}$ levels might compensate for the increase of cell proliferation induced by carcinogen administration since $p27^{Kip1}$ is an inhibitor of cyclin dependent kinase, which blocks progression of the cell cycle (18, 19). However, the increase of $p27^{Kip1}$ was not sufficient to suppress the elevated cell proliferation associated with the corn oil/butyrate group since the cell proliferation was higher compared to the fish oil/butyrate group.

Butyrate may produce conflicting effects on the growth of normal versus cancerous colon cells through its impact on acetyl CoA/histone acetyltransferases (HATs) or as a histone deacetylase (HDAC) inhibitor (45). In normal colon, butyrate acts as a primary energy source and does not inhibit cell proliferation by epigenetic changes via activation of acetyl CoA/HATs. In contrast, in cancer cells butyrate slows cell proliferation but induces differentiation and apoptosis by functioning as a HDAC inhibitor. Further studies are needed to examine the interaction of butyrate with lipid sources with respect to histone acetylation and cell kinetics.

Collectively, data from our study indicate that an enhanced early coordinated response to carcinogen may be one mechanism by which fish oil and butyrate protect against colon tumorigenesis. Our data also suggest that the effects of butyrate may depend in part on the type of fat in the diet. This may partly explain the controversy and inconsistency of butyrate effects on cell cycle kinetics and colon cancer development across in vivo and in vitro studies. Although our data do not address the cellular modifications in the colon that contribute to tumorigenesis, such as epigenetic modifications or stem cell-specific mutations, it does describe outcomes of those modifications and global changes in one form of DNA mutations. The ability of this combination of nutrients to alter global and gene-specific epigenetic states (20, 41, 46) at various stages of tumorigenesis and to modulate downstream events such as proliferation and apoptosis indicate the involvement of multiple mechanisms that contribute to risk reduction. However, further studies are needed to investigate the impacts of fish oil and butyrate on colon adult stem cell damage and epigenetic state.

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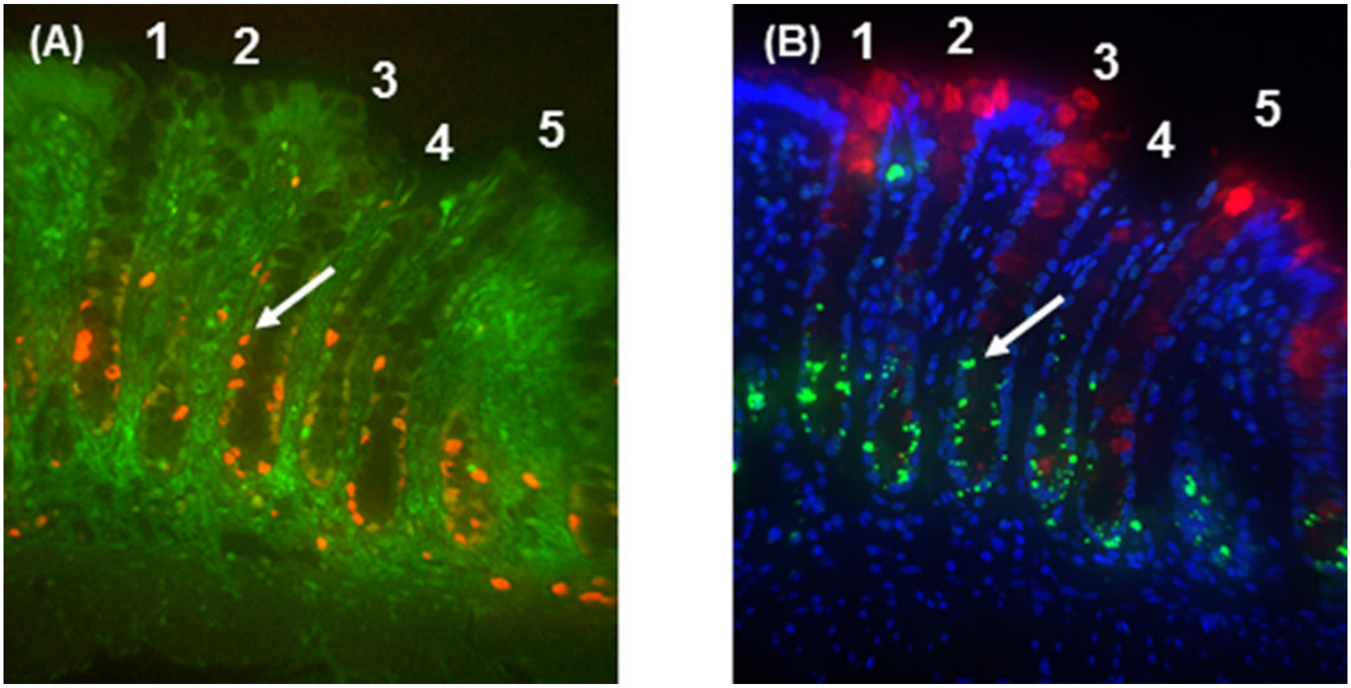

Fig. 1.

Colocalization photomicrograph (400×) of proliferation, p27^{Kip1}, differentiation and apoptosis in serial sections. A: Proliferation (Ki-67, orange) was predominantly localized to the lower part of the crypts and p27^{Kip1} (green) staining was localized in the nuclei of colonic cells within crypts. B: lectin binding (red) was primarily located in the upper part of the crypt and apoptotic cells (green) were found in the lower region of the crypt 9 h after AOM injection. Blue staining represents DAPI counterstaining. Panels A and B are derived from serial sections and crypts 1–5 in panel A are the same crypts shown in panel B. Arrows show the same cell labeled for cell proliferation and p27 ^{Kip1} in panel A, and apoptosis in panel B.

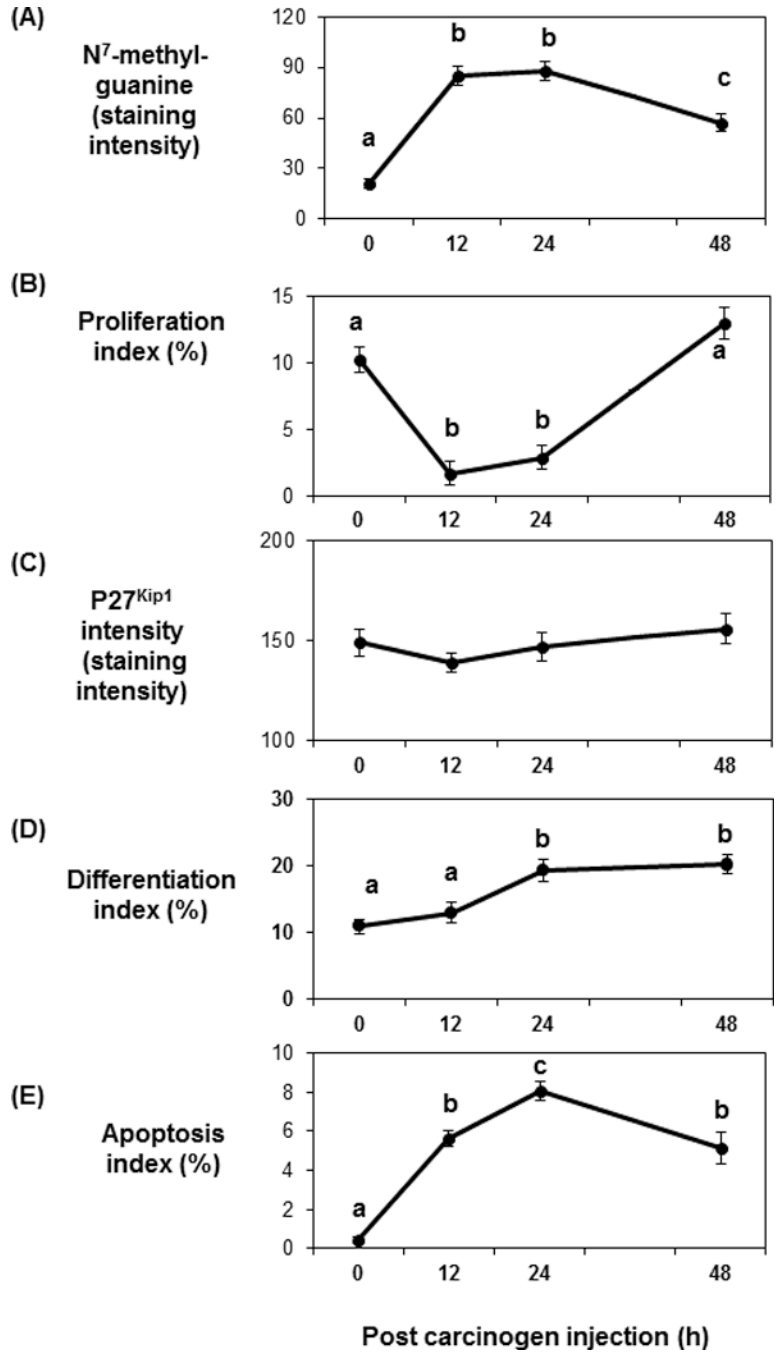

Fig. 2.

Carcinogen effects on DNA adduct level, cell proliferation, p27^{Kip1}, differentiation and apoptosis over time. A: DNA adduct levels were increased 12 h post-AOM injection and decreased by 48 h after AOM injection ($P < 0.001$). B: Cell proliferation decreased 12 h after AOM injection and increased at 48 h post-AOM injection ($P < 0.001$). C: Carcinogen injection did not affect p27^{Kip1} level. D: Differentiation increased 24 h after AOM injection ($P = 0.014$). E: Maximum apoptosis was achieved at 24 h post AOM injection ($P < 0.001$).

Data are presented as means ± SE. Means without a common letter are significantly different.

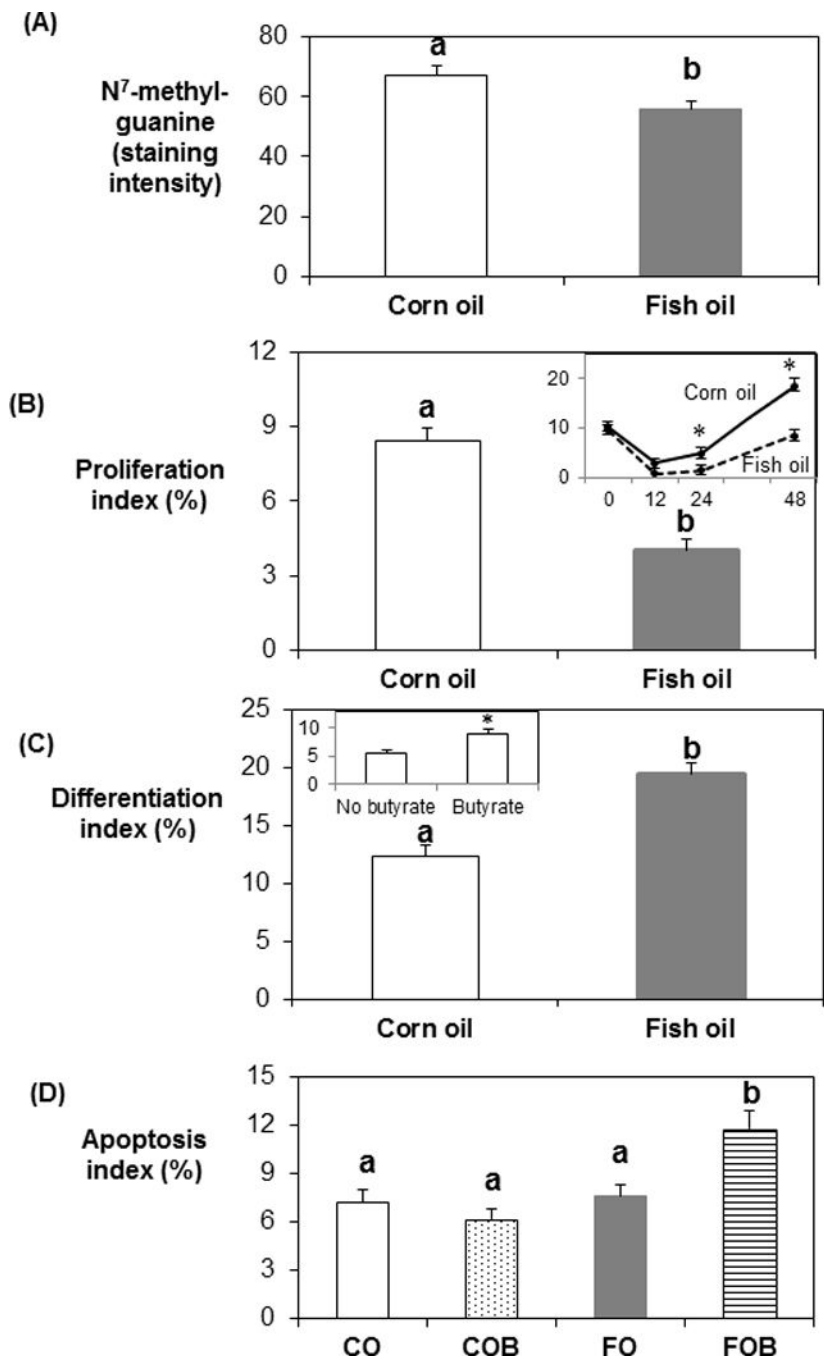

Fig. 3.

Dietary fat effects on DNA adduct level, cell proliferation, p27^{Kip1}, differentiation and apoptosis. Dietary fish oil resulted in lower DNA damage (A, P = 0.001), cell proliferation (B, P = 0.003) and elevated differentiation (C, P = 0.039) compared to the corn oil diet. At 48 h post carcinogen injection, there was a compensatory increase of cell proliferation beyond that observed at 0 h in corn oil-fed rats but not in fish oil-fed rats (P = 0.010, B inset). Butyrate treatment increased differentiation (P = 0.041, C inset). Fish oil/butyrate diet increased apoptosis, relative to the other three groups at 24 h post carcinogen injection (D, P

= 0.039). CO: corn oil; CO/B: corn oil/butyrate, FO: fish oil; FO/B: fish oil/butyrate. Data are presented as means ± SE. Bars without a common letter are significantly different.

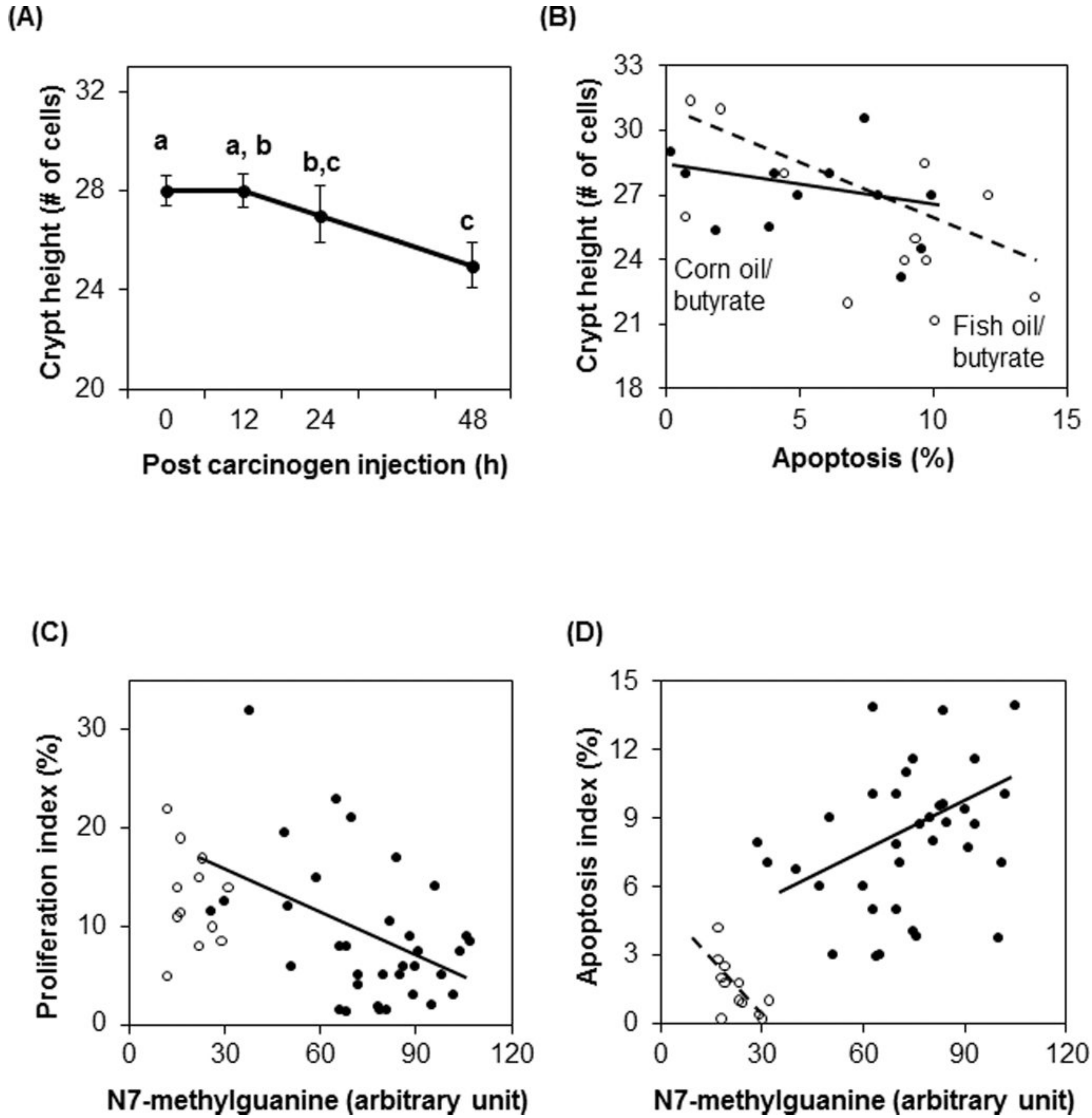

Fig. 4.

Correlation between crypt height and hours post carcinogen injection (A), between crypt height and apoptosis (B), between DNA damage and proliferation (C), and between DNA damage and apoptosis (D). (A) After carcinogen injection, crypt height decreased ($P = 0.011$). Data are presented as means ± SE. Means without a common letter are significantly different. (B) There was a negative relationship between crypt height and apoptosis in the fish oil/butyrate group (correlation coefficient = −0.53, $P = 0.040$) (○,broken line). The correlation between crypt height and apoptosis in corn oil/butyrate-fed rats was not significant (●, solid line). (C) There was no correlation between DNA adduct level and cell proliferation in saline animals (○). After carcinogen injection, there was an inverse relationship between DNA adduct level and cell proliferation (correlation coefficient = −0.42, P = 0.010) (●, solid line). (D) In saline rats, there was an inverse relationship between DNA damage and apoptosis (correlation coefficient = −0.70, P = 0.012) (○, broken line). In contrast, there was a positive relationship between DNA damage and apoptosis after carcinogen injection (correlation coefficient = 0.36, P = 0.033) (*, solid line).

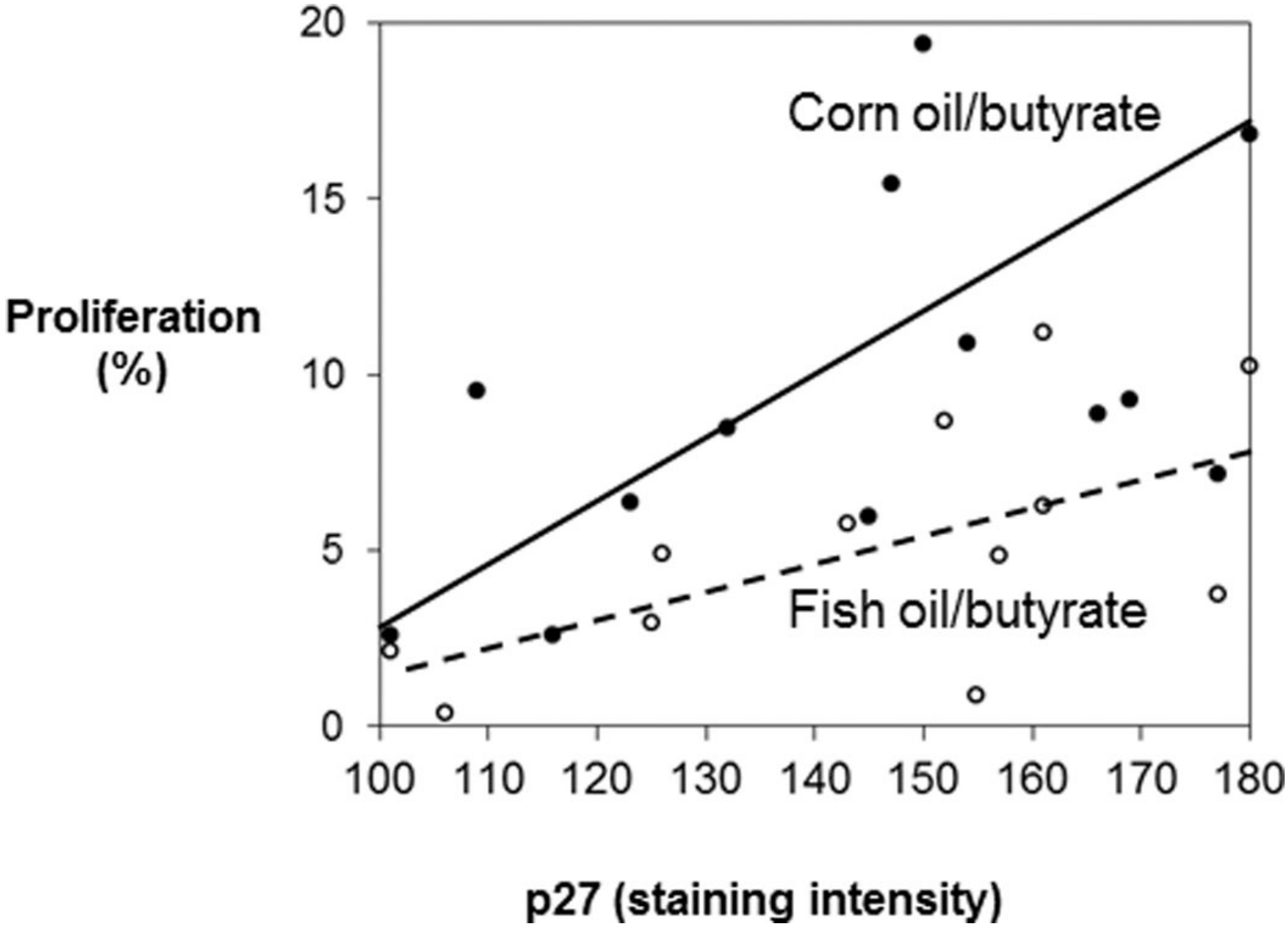

Fig 5.

Correlation between p27^{Kip1} and proliferation as a function of diet. There was a positive relationship between p27^{Kip1} and proliferation in corn oil/butyrate fed animals (correlation coefficient = 0.61, P = 0.035) (●, solid line). The correlation between p27^{Kip1} and cell proliferation in fish oil/butyrate-fed rats was not significant (○, broken line).

Table 1

Composition of experimental diets*

Ingredient	CO (g)	COB (g)	FO (g)	FOB (g)
Dextrose	51.06	51.06	51.06	51.06
Casein	22.35	22.35	22.35	22.35
Cellulose	6.00	6.00	6.00	6.00
Corn Oil	15.00	15.00	3.50	3.50
Fish Oil	0.00	0.00	11.50	11.50
Salt mix, AIN-76A	3.91	3.91	3.91	3.91
Vitamin mix, AIN-76A	1.12	1.12	1.12	1.12
Butyrate pellet	0.00	1.50	0.00	1.50
Total	100.00	101.50	100.00	101.50

*Diets contained equivalent amounts of antioxidants; 26 mg α-tocopherol, 14 mg γ-tocopherol and 2 mg tertiary butylhydroquinone (TBHQ)/100g diet. The butyrate diets were supplemented with Gasto-resistant slow-release butyrate pellets (1.5 g/100g of diet, Valpharma, Serravalle, Italy).