International Journal of
Pathology and Clinical Research

Hypercholesterolemic Diet: Its Effect on Colorectal Precancerous Lesions Induced by Dimethylhydrazine

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Abstract

Objective: Evaluate the effects of dietary cholesterol, without the addition of cholic acid in the initial stage of carcinogenesis and on the bifidobacteria into the colon.

Methods: Rats were grouped according to their diets and dimethylhydrazine treatment (DMH): standard diet (CN); CN + cholesterol 1% (CHOL); CN + DMH (CNDMH) and CN + cholesterol 1%+ DMH (CHOLDMH). After eight weeks of treatment were analyzed weight gain, faecal pH, lipid profile, hepatic and faecal cholesterol, faecal bifidobacteria, cell proliferation and the pre-neoplastic colorectal lesions (aberrant crypt foci (ACF)).

Results: Groups not treated with DMH didn´t develop aberrant crypt. There weren´t significant differences between DMH-groups in the number of ACF and in the multiplicity of ACF/foci. However, cell proliferation evaluated by PCNA-labeling index was significantly higher in the CHOLDMH group when compared to the CNDMH. The highest amount of cholesterol present in the feces of the animals treated with supplemented diet didn´t influence the ACF and number of crypts per focus, but increased cell proliferation.

Conclusion: Diet supplemented with cholesterol or DMH did not influence the amount of bifidobacteria found, suggesting that the mechanism of action of these factors in the initiation of carcinogenesis does not involve change in intestinal microbiota. An increase in the fecal pH was observed in CHOLDMH group, and this association may be a factor that can influence the development of the colorectal precancerous lesions.

Keywords

Cholesterol, Aberrant crypt foci, PCNA, Bifidobacteria

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide. Although its cause is not fully known, genetic susceptibility and diet are major risk factors [1], and fat intake has been the main nutritional influence on its development [2]. However, the involvement of dietary cholesterol in the etiology of the CRC is controversial [3]. Whilst some studies have reported increased risk with high cholesterol intake [4-9], others studies indicate that it may even diminish the development of induced tumors, when administered in the initiation phase of carcinogenesis [10,11]. Cholesterol may influence colorectal carcinogenesis through several mechanisms such as oxidative stress, lipid, glucose and bile acids metabolism or alteration on membrane properties and cell signaling [12-16]. The intestinal microbiota is also recognized as an important player in illness such as CRC. Some studies have suggested that diet can modulate the formation of preneoplastic lesions in the colon, possibly by altering the micro ecology and microbial activities on carcinogenesis [17]. A number of investigations, mainly using in vitro and animal models, have demonstrated a wide range of possible anti-CRC mechanisms by which certain strains from the genera of lactobacilli and bifidobacteria can act. Effects including competitive exclusion of pathogenic intestinal flora, alteration of intestinal microflora enzyme activity, reduction of carcinogenic secondary bile acids, binding of carcinogens and mutagens, increasing short chain fatty acids production, reduction of DNA damage and suppression of aberrant crypt foci formation and modulation of intestinal inflammation are some anti-CRC effects of probiotics on intestinal mucosa [18]. In this context, considering the influence of diet on colon health and the relation between fat intake and the risk of CRC development, this study aims to evaluate the effect of dietary cholesterol (without addition of cholic acid) on the initial phase carcinogenesis and on the amount of colonic bifidobacteria.

Materials and Methods

Rats, diet and dimethylhydrazine (DMH)

This study was approved by animal ethics committee of the Federal University of Santa Catarina (UFSC).

Thirty-two lean adults (30 days old)male Wistar rats (*Rattus norvergicus* variety Albicanus, Rodentia) were randomly divided into four groups in eight colony cages (four rats per cage): standard diet (CN); standard diet + cholesterol 1% (CHOL); standard diet + DMH (CNDMH) and standard diet + cholesterol 1% + DMH (CHOLDMH). After one-week acclimatization period, DMH was administered intraperitoneally (150 mg/kg weigh body), and treatment was initiated with different diets *ad libitum*. To prepare

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the cholesterol diet, the regular chow (Nutrilabor, Brazil) was ground, cholesterol (Sigma-Aldrich, USA) in concentration of 1% (w/w) was added and the mixture was pelletized again. Weight gain analysis was performed by weighing the animals in the first and last day of the treatment. After eight weeks of treatment, stool samples were collected to determine the fecal pH, bifidobacteria, and cholesterol. Blood samples were collected to analyze the lipid profile. The animals were euthanized by exsanguination. Colons were removed for histological analysis. Livers were removed, weighed and the hepatic cholesterol was determined.

Amount of bifidobacteria and fecal pH

Fresh feces were collected from each animal separately. Each animal was placed in an empty cage and was awaited until it evacuate. Feces were weighed and diluted in sterile deionized water for pH determination. Each sample was also weighed and diluted in 9 ml of 0.31 mM phosphate buffer. From this dilution (10-1), serial dilutions were performed up to 10-7 and 100 μl of each dilution was spread plated on selective medium for bifidobacteria BIM-25 [19]. The plates were incubated at 37°C for 72 hours under anaerobic conditions (Anaerobac system, Probac, São Paulo, São Paulo, Brazil). At a later stage, the bacterial population (bifidobacteria) count was calculated as colony-forming units (CFU) per gram of fecal sample. Gram stain, catalase test and fructose-6-phosphate phosphoketolase (F6PPK) test according to Orban and Patterson [20] were performed to confirm *Bifidobacterium* spp. genus.

Biochemical parameters

Hepatic and fecal cholesterol measurements were performed according to Melo *et al.* [21]. Serum triglycerides, total cholesterol and (HDL)-cholesterol levels from each animal were measured using commercial enzymatic kits (Labtest, Montes Claros, Minas Gerais, Brazil).

Histological Analysis

Distal colon was cut, washed with 0.9% saline solution, opened longitudinally and fixed in buffered formalin 10% (pH 6.9-7.1) for 24 hours. Part of the distal colon was stained with methylene blue (0.02%) for five minutes immediately prior to analysis. For each part, 50 sequential fields were analyzed in microscope (100x magnifications) and the number of aberrant crypt foci (ACF) was counted and multiplied by foci (expressed as aberrant crypts per focus (AC/ACF). ACF were characterized by thick epithelial layer which stains more intense with methylene blue, luminal opening elongated, tortuous and increased in relation to the surrounding normal crypts [22-23].

Another part of the distal colon was dehydrated, diaphanized,

embedded in paraffin to obtain 3 μm longitudinal histological sections. To estimate colonic cell proliferation in all animals, the longitudinal sections were immunostained for proliferative cellular nuclear antigen (PCNA) using an antibody against PCNA (Thermo Scientific, Pittsburgh, Pennsylvania, USA) [24-26]. The labeling index (LI) was determined as the ratio of the number of stained nuclei and total number of nuclei along each crypt. Ten crypts were counted per section/animal (x400). Rat colon (Wistar, male) specimens were used as a positive control and as negative control primary antibody suppression.

Statistical Analysis

The data was analyzed using GraphPad Prism V.4 (GraphPad Software, Inc., San Diego, CA). Verification of symmetric distribution of data was performed using Levene's normality (Shapiro-Wilk test). The statistical significance of differences between data means was determined by using analysis of variance one way (ANOVA), followed by post hoc testing by Tukey's test. A value of $P < 0.05$ was considered as statistically significant. Results are given as means ± standard error, n = 8.

Results

Body weight gain

Initial and final weight, weight gain, serum triglycerides, total cholesterol, HDL-cholesterol, hepatic cholesterol, fecal cholesterol, bifidobacteria and pH, number of ACF/field, AC/ACF and PCNA labeling index are shown in table 1.

There were no significant differences between groups in initial weight, gain weight or final weight. Likewise, cholesterol diet and DMH alone did not influence the amount of bifidobacteria and fecal pH.

The serum levels of triglycerides in the CNDMH and CHOLDMH groups were significantly lower than the respective groups treated with the same diet without DMH. However, the addition of 1% cholesterol in the diet was not sufficient to increase total cholesterol levels. Moreover, fecal cholesterol level in CHOL group was significantly higher than the CN group. The same finding was observed when compared CHOLDMH and CNDMH groups.

Among the precancerous lesions markers, no significant differences in ACF/field and AC/ACF were observed between groups (Table 1 and Figure 1). Otherwise, PCNA labeling index was significantly higher in CHOLDMH than the CNDMH group (Table 1).

Discussion

Interactions between fatty acids and intestinal microbiota have

Table 1: Results of serum, fecal, liver and colonic samples analysis from different experimental groups.

	CN	CNDMH	CHOL	CHOLDMH
Initial weight (g)	180 ± 6	166 ± 7	147 ± 7	152 ± 3
Final weight (g)	314 ± 9	297 ± 16	291 ± 16	296 ± 7
Gain weight (g)	134 ± 7	149 ± 7	146 ± 8	144 ± 7
Triglycerides (mg/dl)	82 ± 4	59 ± 4*	85 ± 7	58 ± 5*
Total cholesterol (mg/dl)	128 ± 8	123 ± 8	111 ± 5	105 ± 7
HDL-cholesterol (mg/dl)	36 ± 3	37 ± 2	36 ± 2	34 ± 1
Hepatic cholesterol (mg/dl)	31 ± 2	37 ± 3	35 ± 3	33 ± 2
Fecal Cholesterol (mg/dl)	34 ± 5	24 ± 3	66 ± 3**	69 ± 5++
Bifidobacteria (UFC/g)	$4.35 ± 3.39×10^{10}$	$8.37 ± 3.10×10^{10}$	$9.39 ± 3.64×10^{10}$	$4.48 ± 2.81×10^{10}$
Fecal pH	7.0 ± 0.3	7.2 ± 0.3	6.5 ± 0.1	7.1 ± 0.3*
PCNA (%)	5.76 ± 0.91	16.20 ± 1.70	8.44 ± 0.23	20.48 ± 0.37++
ACF/field	0	0.68 ± 0.16	0	0.41 ± 0.50
(AC/ACF)	0	2.52 ± 0.33	0	2.11 ± 0.21

Results are expressed as mean ± standard error. * p < 0.01, compared to the group treated with the respective diet without DMH; **compared to CN group; ++compared to CNDMH group. Standard diet (CN); standard diet + cholesterol 1% (CHOL); standard diet + DMH (CNDMH) and standard diet + cholesterol 1%+ DMH (CHOLDMH). PCNA: proliferating cell nuclear antigen; ACF: Aberrant crypt foci; and AC/ACF: aberrant crypt/aberrant crypt foci.
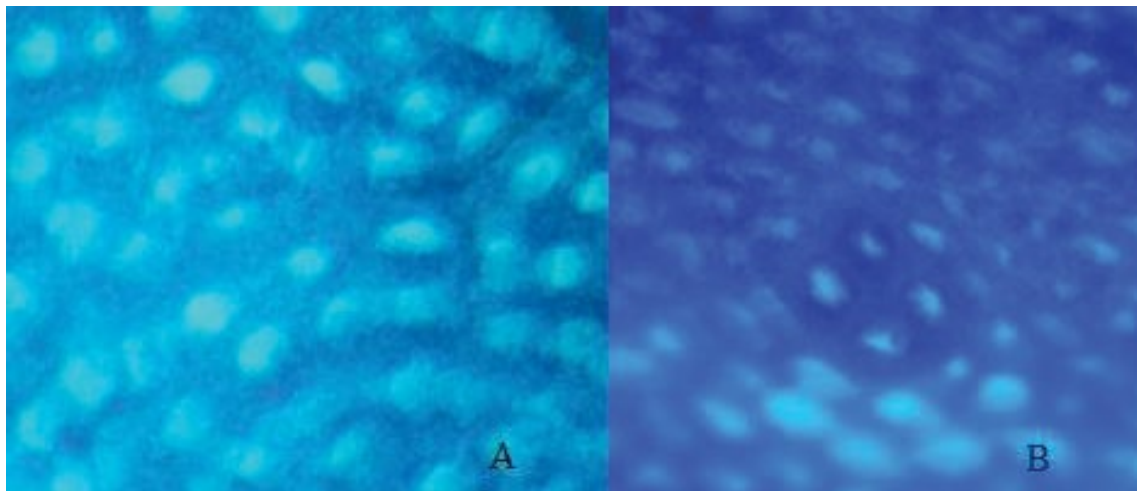
Figure 1: Photomicrograph of colon stained with methylene blue.
A. Microscopic field without ACF; B. Microscopic field with ACF (aberrant crypt foci) with 4 AC (aberrant crypt). 100× magnification.

shown to be related to colon cancer. Some fatty acids, but not all, are potent antimicrobial agents and some bacteria exhibit different sensitivities to distinct fatty acids [27]. These data suggest that fatty acids may influence the biodiversity of the intestinal microbiota and the CRC. In this regard, it is important to evaluate the effect of cholesterol, which is also a common dietary lipid, on colorectal carcinogenesis and bifidobacteria.

After eight weeks of treatment with different diets, triglycerides level was similar between groups not treated with DMH, but was lower in DMH treated groups. Although the mechanism by which DMH reduce triglycerides level is not known, this fact was reported by Nauss et al. [28] to evaluate the effect of the amount of dietary lipids on DMH-induced colonic tumorigenesis in rats for four weeks. On the other hand, Barton et al. [29] observed increased triglycerides levels in late stages of colon carcinogenesis induced by DMH.

Experimentally, diet plus 1% cholesterol was not enough to significantly increase the serum cholesterol and liver cholesterol levels. This can be explained by the higher conversion to bile acids in the liver and higher fecal cholesterol excretion [21,30]. Endogenous cholesterol is excreted into bile as free cholesterol or converted by the liver into bile salts [31]. The highest fecal cholesterol level in groups treated with cholesterol diet may have been due to increased excretion or probably due to poor absorption of dietary cholesterol, not causing, thereby, hypercholesterolemia.

Furthermore, *in vivo* studies using rodents, hypercholesterolemia is usually achieved with addition of cholic acid in the diet enriched with cholesterol [32,33].

Evidences have shown that the greater contact of harmful agents such as bile salts, with the colonic mucosa may determine important epithelial alterations, and may lead to the development of colorectal cancer [34,35]. Contrary to other studies, our study was conducted without addition of cholic acid in the diet to assess the effect of cholesterol alone on the development of ACF and bifidobacteria content.

The experimental model of quantifying ACF is considered an excellent parameter to evaluate the protective or risk factor for colorectal cancer [36]. In this context, it was observed that the group not treated with DMH did not develop aberrant crypt. This indicates that the cholesterol alone, in this concentration on diet, did not exert initiator action of colon carcinogenesis. The presence of higher content of cholesterol in the feces of the animals treated with cholesterol did not influence the amount of ACF and the number of crypts per focus, perhaps due to the short treatment period (60 days). However, false-positive or false-negative results can occur due to various factors of the natural process of colonic carcinogenesis [37].

On the other hand, the highest amount of fecal cholesterol increased the cell proliferation, which is an important prerequisite for colorectal carcinogenesis [37,38]. This result corroborates with studies that reported increased risk of CRC in diets with high cholesterol content [4-7] and it might be due to the high production of bile acids in the intestine.

Therefore, although no significant difference in the parameters ACF/field and AC/ACF, the largest cell proliferation (PCNA) observed in groups treated with cholesterol, and possibly caused by increased of fecal cholesterol, will not allow to discard the hypothesis that a high-cholesterol diet can influence the colorectal cancer etiology if analyzed in a later stage of this cancer. This hypothesis corroborates with Tseng et al. [32] that suggested that cholesterol is a non-carcinogenic agent that can potentiate the carcinogenicity of DMH through increased lipid peroxidation and decreased peroxidase activity of the colon. These researchers when examine the influence of hypercholesterolemic diet (0 to 2%) and cholic acid (0.25%) for 18 weeks observed that rats treated with DMH, cholic acid and higher cholesterol concentration (2%) in the diet, develop 50% more colon adenomas than rats just treated with DMH.

The results showed that dietary cholesterol or DMH does not influence the amount of bifidobacteria. It can suggest that the mechanism of action of these factors in the initiation of carcinogenesis does not involve change in intestinal microbiota as proposed by others researchers who observed a reduction on bifidobacteria in experimental colorectal carcinogenesis [39,40]. Although no effect on the amount of bifidobacteria, DMH showed a tendency to cause an increase in fecal pH, which was significant in CHOLDMH and this association may be a factor which can influence the CRC development, in agreement with other studies [41].

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