Decrease of colonic dysplastic lesions induced by 1,2–dimetylhydrazine in butyric acid supplemented rats

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Abstract

Catuogno, M.S.; Montenegro, M.A.; Sánchez Negrette, M.; Ramirez, G.V.: ***Decrease of colonic dysplastic lesions induced by 1,2–dimetylhydrazine in butyric acid supplemented rats.*** *Rev. vet. 22: 1, 13–18, 2011.* The butyric acid is considered as an anticancerigenous compound in several models of experimental carcinogenesis. It is one of the end products of the non–gaseous fermentation carried out by the microbial anaerobic flora of the mammalian large bowel and rumen. The milk and dairy products are the main source of butyric acid among the animal products. The aim of this study was to determine the antitumorigenic effect of butyric acid on the development of dysplastic lesions, considered as unequivocal histologic markers of colon cancer in its initial state, in 1,2–dymethylhidrazine–treated rats. Six–week–old Wistar male rats with average body weight of 160 g were used. Butyric acid was administered diluted in drinking water at the dose of 4 mg/ml for 26 weeks. Dysplastic lesions in the large bowel were induced by a weekly subcutaneous injection of 1,2–dymethylhidrazine during 17 and 5 times to respective sub–groups. Less incidence of dysplastic lesions was observed in butyric acid–supplemented groups, both in sub–group with 17 inoculations and in the sub–group with 5 inoculations, compared to control sub–groups (without supplementation). The dysplastic lesions also displayed variation in the mucin content depending on the severity of the dysplasia. We conclude that the use of butyric acid as an antitumorigenic compound, would be the reason for the marked decrease on the development of precarcinomatous lesions, considered as important precursors of colon carcinomas.

Key words: rat, dysplastic lesions, colon cancer, butiric acid, dymethylhidrazine.

Resumen

Catuogno M.S.; Montenegro M.A.; Sánchez Negrette M.; Ramirez G.V.: ***Disminución de lesiones displásicas colónicas inducidas por 1,2–dimetilhidrazina en ratas suplementadas con ácido butírico.*** *Rev. vet. 22: 1, 13–18, 2011.* El ácido butírico es considerado como agente anticancerígeno en varios modelos de cáncer experimental. Constituye uno de los productos finales de la fermentación no gaseosa producida por la flora microbiana presente en intestino grueso y rumen de mamíferos. Entre los productos de origen animal, la leche y los productos lácteos constituyen una de las principales fuentes de ácido butírico. Nuestro objetivo fue evaluar el efecto anticancerígeno del acido butírico sobre el desarrollo de lesiones displásicas, consideradas marcadores histológicos de cáncer de colon en sus estadios iniciales, en ratas tratadas con el carcinógeno 1,2–dimetilhidrazina. Fueron utilizadas ratas Wistar, machos con un peso promedio de 160 g a las cuales se les administró ácido butírico puro diluido en el agua de bebida a la concentración de 4 mg/ml, durante 26 semanas. Las lesiones displásicas en el intestino grueso fueron inducidas mediante una inyección subcutánea semanal de 1,2–dimetilhidrazina durante 17 y 5 semanas a los respectivos grupos. En los lotes suplementados con ácido butírico se observó menor incidencia de lesiones displásicas tanto en el grupo tratado con 17 inoculaciones como en el grupo tratado con 5 inoculaciones, comparado con los grupos controles que no recibieron ácido butírico. Las lesiones displásicas también mostraron variación en el contenido de mucina dependiendo de la severidad de la displasia. Concluimos que la utilización de ácido butírico como agente anticancerígeno sería responsable de la marcada disminución del desarrollo de lesiones precarcinomatosas, consideradas como importantes precursores del carcinoma de colon.

Palabras clave: rata, lesiones displásicas, cáncer de colon, ácido butírico, dimetilhidrazina.

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INTRODUCTION

Experimental models in laboratory animals give an excellent chance to study tumor processes and pre–neoplastic lesions. The dysplastic lesions are precancerigenous or precarcinomatous lesions considered as unequivocal histological markers in the initial states [12, 17] of colon cancer [26]. These lesions are described as lonely entities that can appear in the colonic mucosa, forming part of both adenomatous [17] or carcinomatous lesions [26]. They are also described as aberrant crypt foci (ACF) [22]. The ACF are considered to be colon cancer preneoplastic changes based on their cellular, molecular and morphological features [3, 4]. Besides, some ACF with dysplasia are diagnosed as microadenoma [20], adenoma [17] or *in situ* carcinoma [26] indicating sometimes that ACF presents preneoplastic lesions [27].

Dysplastic lesions are characterized by proliferative phenomenon, gland crypts with enlarged lumens, marked cell basophilia, goblet cells depletion and polarity loss [15]. Another features mentioned include increase nuclear size, nuclear pseudostratification, variable amounts of mitoses and regular or irregular overstruck tubular structures and ramifications [1].

In relation to molecular pathways, it is known that dysplastic lesions referred as adenomatous crypts or microadenomas, frequently show loss of heterozygosity on 5q, the adenomatous polyposis coli locus [11, 21]. The ACF are considered to be precursors of adenomatous polyps that are thought to be predecessors lesions of colonic carcinoma. The transition from adenoma to a high grade dysplasia seems to involve the P 53 gene, considered the guardian of the genome [10].

Epidemiological studies in human beings link the dysplastic lesion origin and colonic cancer development with fatty foods. The involved fats are from animal source, mainly the saturated ones, which are found in red meat and dairy products, as well as the hydrogenated ones, taken out from vegetable oil. At least it explains in part the highest rate of those colon cancers in some south American countries [23, 28–30]. Cow milk presents several compounds considered as potential inhibitors of the carcinogenesis process. Such compounds include sphingomyelin, conjugated linoleic acid, vitamin A, β–carotene, vitamin D, calcium and butyric acid [23].

In our laboratory, the effects of a dairy diet in the experimental colon carcinogenesis have been studied. In those experiments we observed fewer amounts of tumours in rats feeded with skimmed milk powder than in animals feeded with whole milk powder [23]. The same effects have also been noticed in other studies using cow milk and buffalo milk [24].

Epidemiological studies in humans and researches in rats have demonstrated that a fiber rich diet reduces colon carcinoma incidence and mortality [5]. In mammalians, the microbial anaerobic flora of the large bowel and rumen produce short chain fatty acids (SCFAs), which are the acetic, propionic and butyric acids considered as the main non–gaseous fermentation end products [18].

Butyric acid (BuAc) is a short chain fatty acid derived from fermentation of non–digestible carbohydrates in the colon. Such acid blocks the tumour cell proliferation in the colon cancer and that is the reason of why BuAc is thought to be the main protective compound of a fiber rich diet in the colonic carcinomas [5]. The butyrate is the preferred energy source for the colonic mucosa and has been implicated in protection against colitis and colorectal cancer [18].

Some properties of the SCFAs are quick and easy absorption by the intestinal mucosa, and relative high caloric content, are metabolized by colonocytes and hepatocytes. They stimulate colonic absorption of sodium and water, and have a trophic effect on the intestinal mucosa. The fermentative production of SCFAs has been admitted as a main mechanism in the ruminant intestinal digestion, whereas in human beings this remains unclear. Because of this, the interest in SCFAs production and their effects has been growing in the last 10 years [7].

All three SCFAs tend to slow proliferation, promote brush border enzyme activity, and inhibit both adhesion to and motility across the type I collagen matrix [2]. They stimulate proliferation of normal crypt cells too, but butyrate and, in a lower degree, propionate inhibit colon cancer cell lines growth. At molecular level, butyrate causes histone acetylation and induces apoptosis and regulates the expression of various oncogenes [25].

The purpose of this report was to determine the antitumorigenic effect of butyric acid, a common compound found in dairy products and which is naturally produced as a result of the fermentation of non–digestible carbohydrates, on the dysplastic lesions development in the colon of 1,2–dymethylhidrazine (DMH) treated rats.

MATERIAL AND METHODS

Animals: six–week–old Wistar male rats with an average body weight of 150 g, were purchased from Facultad de Ciencias Veterinarias (UNNE, Corrientes, Argentina), and housed at individual cages in a temperature–controlled room at 21ºC. The animals were given a nutritionally adequate diet and drinking water *ad–libitum* and randomly divided into two main groups and sub–groups at the following manner:

Control Group (without DMH treatment) formed by: *Sub–group 1* (without treatment n=10) and *Sub–group 2* (supplemented with BuAc n=20).

Experimental group (with DMH treatment) formed by: *Sub–group 3* (treated once a week 17 times with DMH n=20), *Sub–group 4* (treated once a week 17 times with DMH + BuAc n=20), *Sub–group 5* (treated once a week 5 times with DMH n= 20), *Sub–group 6* (treated once a week 5 times with DMH + BuAc n = 20).

Butyric acid: BuAc was administrated in the drinking water at the dose of 4 mg/ml during 26 weeks to sub–groups 2, 4 and 6. This dose was chosen taken into account that the cow milk contains 0.959 mg of

BuAc/ml. Besides an adult rat drinks between 20 and 30 ml of milk taking 19 to 29 mg of BuAc. Thus, the animals consumed a higher concentration of BuAc than that found in other foods such as cow milk.

1,2–dymethylhidrazine: dysplastic lesions were induced by a weekly subcutaneous injection of DMH during 17 weeks to sub–groups 3 and 4; and 5 weeks to sub–groups 5 and 6, at the dose of 20 mg/kg of body weight. Two weeks before the first DMH treatment pure BuAc 99% (Sigma–Aldrich) was administered in the drinking water to sub–groups 2, 4, and 6. The BuAc supplement was given until the sacrifice performed 26 weeks after the beginning of the experiment. The DMH solution used for injection was prepared with 400 mg of DMH (symmetrical dimethylhydrazine dihydrochloride, Sigma Chem.Co) dissolved in 100 ml of demineralized water containing 37 mg of ethylene–diamine tetraacetic acid. The pH was raised to 6.5 with sodium hydroxide. The solution was prepared every week just before the inoculation.

General observations: every animal was daily examined looking for physical and behaviour. The body weights were weekly recorded since the beginning of the experiment till the sacrifice.

Sacrifice: after 26 weeks, the animals were sacrificed. Each one was necropsied, paying particular attention to the large bowel, which was removed and opened along its longitudinal axis. The entire large bowel was then fixed in 10% neutral buffered formalin during 24 hours.

Study of the samples: the cleaned and fixed large bowels were divided into 4 segments for study: rectum, distal colon, proximal colon, and cecum. The bowels were cut in segments and the swiss–roll technique was performed. Then each segment was processed by the histological classic technique, embedded in paraffin wax, cut at 5 µm and stained with hematoxilin–eosin (HE), periodic acid–Schiff (PAS) and alcian blue (AB) pH 2.5. Histological slides of each large bowel segments (cecum, proximal colon, distal colon and rectum) were analyzed to detect, locate and typify dysplastic lesions. Dysplasic lesions were classified following a classical criterion [26] :

• *ACF with mild dysplasia*: exhibit enlarged nuclei, loss of mucin and some nuclear stratification.

• *ACF with moderate dysplasia*: exhibit pleomorphic nuclei, extensive nuclear stratification and greater loss of mucus production compared with mildly dysplastic ACF.

• *ACF with severe dysplasia*: or carcinoma *in situ*, exhibit highly pleomorphic nuclei that are greatly stratified, a higher degree of loss of mucin, and occasionally increased mitotic activity [26] .

The dysplastic lesion is defined as a "precarcinomatous change" and as "an unequivocal neoplastic alteration of the colonic epithelium" [19] .

Statistical Analysis: to calculate the statistical differences on the lesions incidence it was used a χ^2 test and a simple one way analysis of variance (ANOVA) to compare the weight gain for each treatment group.

Table 1. Frequency and location of dysplastic lesions in the segments of the large bowel. Sub–groups treated with 17 inoculations of DMH.

sub–group	DMH – 17	AcBu + DMH – 17
number of rats	n = 20	n = 19
number dysplastic lesions	117	50
rectum	2 (1.71%)	2 (4%)
distal colon	68 (58.12%)	34 (68%)
proximal colon	34 (29.06%)	12 (24%)
cecum	13 (11.11%)	2 (4%)

( ) Percentage over total of dysplastic lesions in each sub–group.

Table 2. Frequency and location of dysplastic lesions in the segments of the large bowel. Sub–groups treated with 5 inoculations of DMH.

sub–group	DMH – 5	AcBu + DMH – 5
number of rats	n = 20	n = 20
number dysplastic lesions	33	14
rectum	1 (3.03%)	1 (7.14%)
distal colon	25 (75.75%)	9 (64.28%)
proximal colon	4 (12.12%)	2 (14.28%)
cecum	3 (9.09%)	2 (14.28%)

( ) Percentage over total of dysplastic lesions in each sub–group.

RESULTS

Throughout the experience were not recorded any sub–group significant weight differences. The χ^2 test showed a significant difference on the lesion incidence. The dysplastic lesions were found in most of the DMH treated animals in cecum, proximal colon, distal colon and rectum. A lower incidence of dysplastic lesions was revealed in both AcBu supplemented groups: sub groups with 17 inoculations (n=50) and the other with 5 inoculations (n=14). While both sub groups just treated with DMH (the 17 inoculations subgroup and 5 inoculations one) showed 117 dysplastic lesions and 33 lesions, respectively.

The amount and location of dysplastic lesions are registered on Tables 1 and 2. The dysplastic lesions involved 1 to 10 crypts and were located on the surface, in the middle or filling the whole thickness of the mucosa. Such lesions exhibited marked cell basophilia and prominent nuclei with an evident nucleoli. Depletion or absence of goblet cells, cell stratification and loss of polarity as well as gland shoot structures. This kind of lesion presented complete absence of mucin production (Figure 1).

The dysplastic foci displayed variation in the mucin content depending on the dysplasia severity. In the mild dysplastic lesions were observed a same depletion of PAS+ mucin and AB+ mucin whereas changes in the distribution were observed. PAS+ mucin were distributed homogeneously inside the cytoplasm. The PAS+ mucin was distributed with an uniform pattern inside

the cell cytoplasm (Figure 2) but AB+ mucin was located mainly on the apical surface of the cells (Figure 3). Few lesions with moderate dysplasia showed a slight tendency to loss PAS+ mucin. The severe lesions lost the production of PAS+ mucin as well as AB+ mucin.

DISCUSSION

The BuAc plays an important role in cell growth regulation, it is considered to be an inhibitor of cell proliferation and to strongly induce *in vitro* differentiation for a wide variety of neoplastic cells [6], including mammary gland, large bowel, rectum, liver, cervix and ovary [5, 12, 16].

Furthermore BuAc induces apoptosis by modulation of pro–apoptotic and anti–apoptotic gene expression as well as liberation of citochrome C, a pro–apoptotic enzyme, from the mitochondria [16]. This type of apoptosis is considered p53 independent and there are evidences that it occurs as a final stage of the differentiation process (the apoptosis p53 dependent in response to DNA damage occurs through another pathway opposite to the terminal differentiation) [6]. In another experience the BuAc arrested the growth of neoplastic colonocytes in G1 phase of cell cycle and blocked the neoplastic cell proliferation by inhibition of proto–oncogenes *ras*, *src* and *c–myc* and induction of proto–oncogenes *c–fos* and *c–jun* [5].

Cyclins, cyclin–dependent kinases (cdk) and cdk inhibitor proteins are some of the molecular pathways through which BuAc causes cell cycle arrest and differentiation to prevent the cell proliferation [16]. Some authors attribute BuAc anti–inflammatory effects because of its ability to induce enzymes (i.e. transglutaminase) able to promote the mucosal growth [7]. In spite of the properties previously mentioned, some studies do not support a chemopreventive effect. There are opinions that disagree respect to differences in the *in vivo* and *in vitro* studies, time of BuAc administration as well as the amount and source of BuAc used [16].

Experimental works have used sodium butyrate as a BuAc source; it was administered by luminal instillation and oral consumption at high concentration. Some results have shown promoter effects whereas other ones present anticancerigenous effects [13]. Trying to demonstrate that the responsible of the colon cancer increase in rats was sodium and not the butyrate consumption, some studies tried a tributyrin (a neutral short chain fatty acid triglyceride) supplementation. In that opportunity differences in focal dysplastic areas or colonic tumours have not been observed [8].

In the present experience, using pure BuAc diluted in drinking water, without sodium and esterification, was observed an important decrease of dysplastic lesions, considered pre–neoplastic lesions and histological markers of colon cancer, compared with the two-fold found in sub–groups DMH–treated without BuAc supplementation.

The decrease of precarcinomatous lesions development could due to the pure AcBu use at high concentrations during a long time exposure. These observations

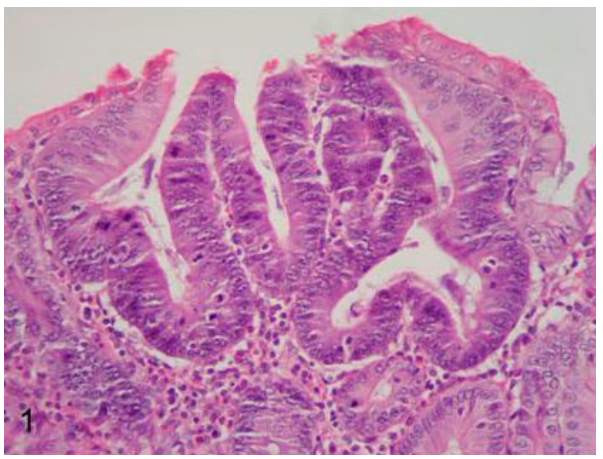

Figure 1. Colon. Severe dysplastic foci located on the mucosa surface. Pseudopapilar formation, loss of mucin production, hyperchromatic nuclei, numerous mitotic figures and stratified nuclei. HE, 10x.

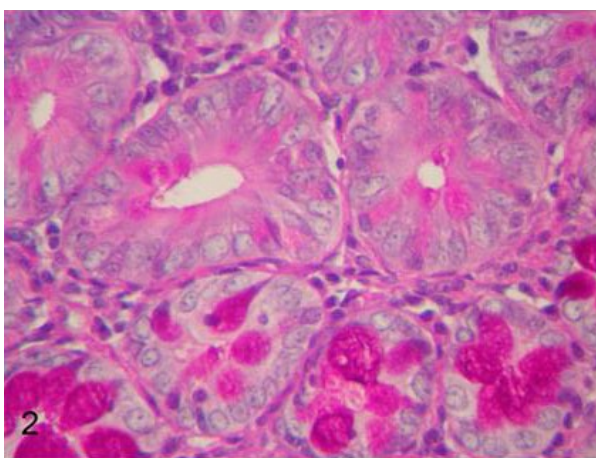

Figure 2. Colon. Crypt foci with mild dysplasia. PAS staining demonstrate a remarkable mucin depletion compared with surrounding crypts (normal ones are shown on the bottom of the picture). PAS, 40x.

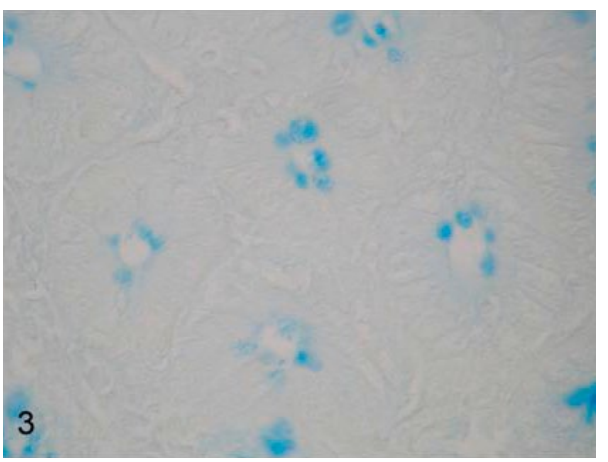

Figure 3. Colon. Crypt foci with mild dysplasia. AB pH 2.5 staining showing the AB+ mucin is distributed almost exclusively in the apical part of the colonocyte. AB, 40x.

agree with investigations which support the fact that the BuAc is an inhibitor of cell proliferation [6] , as well as tumor cell proliferation in colon cancer [5] .

Respect to the distribution of dysplastic lesions in large bowel segments, we observed less development of lesions in cecum of sub–groups treated with more DMH doses. That observations are coincident to experimental works made with molybdenum and tungsten, as well as with dairy diets, in which cecum was the large bowel segment that shown less amount of tumours [14, 23] . Taking into account the results obtained by our research group, we could consider that the BuAc would be the responsible for the marked development decrease of precarcinomatous lesions considered as important precursors of colon carcinomas.

Besides, we observed AB+ mucin predominance in lesions with moderate dysplasia, as well as total mucin depletion in severe lesions and carcinomas according with other works, reflecting a possible early malignant transformation [9] .

At the same time in the present study we observed some mild dysplastic lesions with an irregular distribution of the mucin. A few lesions presented an homogeneous distribution of PAS+ mucin inside the cell, whereas AB+ mucin had a tendency to locate mainly on the apical area of the cells. This findings should be study further in the future to determine its importance in the early diagnosis of lesions with malignant transformation potential.

This data support the theory that several diet components play an important role on the development of both cancer precursor lesions and colon cancer in rats.

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