



Original/Cáncer

Defatted flaxseed meal prevents the appearance of aberrant crypt foci in the colon of mice increasing the gene expression of p53

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Abstract

Objective: The goal of this study was to investigate the preventive effects of defatted flaxseed meal on the appearance of aberrant crypt foci (ACF) in mice treated with 1,2-dimethylhydrazine (DMH).

Materials and Methods: ACF were induced by intra-peritoneal administration of 20 mg/kg body weight of DMH for 8 weeks. The animals were divided into three treatments (n=8): AIN93M diet without fibers + DMH (C-); AIN93M diet with defatted flaxseed meal (LIN); and AIN93M diet with defatted flaxseed meal + DMH (LINCA), for 15 weeks. The technique RT-PCR was used to evaluate the expression of p16, p21, p53, cyclin D1, and cyclin E in the distal colon. In addition, flow cytometric analysis of CD4 and CD8 spleen cells, the quantification of short-chain fatty acids (SCFA) in stool, the quantification of *Bifidobacterium spp.*, *Clostridium spp.* in feces.

Results: LIN and LINCA showed increased *Bifidobacterium spp.* compared with control (C-). In relation to the weight of the organs, the groups LIN and LINCA showed higher values for the liver and kidney compared with control (C-). Regarding ACF, the group LINCA presented fewer ACF in the middle and distal colon compared with control (C-). When we analyzed ACF with more than three crypts the group LIN and LINCA did not present ACF in the middle and distal segments. LINCA presented increased p53 gene expression.

Conclusion: This finding suggests that defatted flaxseed meal reduces ACF by increasing the expression of p53 and increase *Bifidobacterium spp.*

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Key words: Defatted flaxseed. Aberrant crypt foci. P53 protein. *Bifidobacterium spp.*

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HARINA DE LINAZA DESGRASADA PREVIENE LA APARICIÓN DE FOCOS DE CRIPTAS ABERRANTES EN COLON DE RATONES AUMENTANDO DE LA EXPRESIÓN DEL GEN DE P53

Resumen

Objetivo: El objetivo de este estudio fue investigar el efecto preventivo de la harina de linaza desgrasada en el desarrollo de focos de criptas aberrantes (FCA) en ratones tratados con 1,2-dimethylhydrazina (DMH).

Material y Métodos: FCA se indujo por inyección intraperitoneal de 20 mg / kg de DMH por kilogramo de peso corporal durante 8 semanas. Los animales fueron divididos en 3 grupos (n = 8): dieta sin fibra de la dieta AIN93M DMH + (C); Dieta AIN93M con harina de linaza desgrasada (LIN); dieta AIN93M y la dieta con harina de linaza desgrasada + DMH (Enlace) durante 15 semanas. RT-PCR se utilizó para evaluar la expresión de p16, p21, p53, ciclina D1, ciclina E y el colon distal. Los análisis CD4 y CD8 se realizaron con citometría de flujo, así como la cuantificación presencia de *Bifidobacterium spp.* y de *Clostridium spp.* en las heces. La cuantificación de ácidos grasos de cadena corta (AGCC) fue realizada por el método de cromatografía de gas.

Resultados: LIN y LINCA mostraron un aumento significativo de *Bifidobacterium spp.*, en comparación con el control (C) (p <0,05). Con relación al peso de los órganos, los grupos LIN y LINCA mostraron valores aumentados de hígado y riñón en comparación con el control (C) (p <0,05). Encuanto a la FCA, los grupos FCA LINCA mostró menor en los dos puntos media y distal en comparación con el control (C) (p <0,05). Cuando analizamos FCA con más de tres focos de criptas aberrantes, los grupos LIN y LINCA no presentaron FCA en los segmentos medial y distal, en contraste con el grupo control (p <0,05). LINCA mostró aumento de la expresión del gen p53 (p <0,05).

Conclusión: Estos resultados sugieren que la harina de linaza desgrasada reduce FCA, para aumentar la expresión de p53.

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Palabras clave: Linaza desgrasada. Focos de criptas aberrantes. Proteína p53. *Bifidobacterium spp.*

Introduction

Colorectal cancer (CCR) affects a large population worldwide and is the third most prevalent cancer in the world¹. It occurs most frequently in the distal segments of the colon, and most cases are diagnosed in the advanced stages of the disease, the organs most common presenting metastases are the liver, lung, and lymph nodes².

One of the first indicators of the development of this cancer is the presence of aberrant crypt foci (ACF) or pre-neoplastic lesions³. ACF are lesions characterized by morphologically abnormal crypts on the surface of the colonic mucosa, which have been described primarily in rats and taken as evidence for the future development of the CCR⁴. With growth, aberrant crypts can give rise to visible polyploid lesions and with time, can show morphological changes, becoming less distinct and more dysplastic, and acquiring the phenotype of carcinoma, becoming a metastasis⁴. ACF can be observed and quantified microscopically, and the count of these foci is a marker for the risk of developing colorectal cancer^{5,6}.

Various types of food substances are being investigated for their preventive effect against CCR among them are the fibers correlated daily in the prevention of various diseases of the digestive system including CCR^{7,8}. The relationship between dietary fiber intake and risk of colon cancer has been studied for over 30 years. However, the data are not conclusive. There are many reasons for these inconclusive results, such as the diverse sources and types of fibers, in addition to the various sites of location of colon cancer (different segments), especially in humans⁷. Lifestyle, the use of tobacco and alcohol, and food consumption of different modes are also factors that potentially increase the variables to get the mechanisms of action of substances with carcinogenic effects⁸.

Foods that are high in fiber have proved to be important in the prevention of several diseases⁹. Among them is bran defatted flaxseed, which constitutes a good source of nutrients including fiber and essential fatty acids. The most important fiber fraction, consisting of resistant starches, cellulose, and complex polymers such as lignans¹⁰.

Objective

To clarify its relevance, efficacy, and possible mechanism of action, the objective of this study was to investigate the preventive effect of defatted flaxseed meal on the appearance of ACF in mice treated with DMH (1,2-dimethylhydrazine).

Materials and Methods

Experimental protocol

Forty 12-week-old, male Balb/C mice weighing approximately 25 g, from the Federal University of

Viçosa (UFV), were used. During the experiment, the animals were allocated individual cages that were cleaned daily and maintained in an environment with controlled temperature ($22^{\circ}\text{C}\pm 2^{\circ}\text{C}$), humidity (60–70%), and illumination (12-h light/dark cycles). The animals were divided into three treatments (n=8): AIN93M diet without fibers + DMH (C-); AIN93M diet with defatted flaxseed meal (LIN); and AIN93M diet with defatted flaxseed meal + DMH (LINCA). Animals were given *ad libitum* water and a modified AIN93M diet¹¹, with 10% defatted flaxseed meal added instead of cellulose (a fiber-free diet) for 15 weeks. The experiment was conducted according to the Guiding Principles in the Use of Animals Ethics Committee of the Department of Veterinary Medicine of Federal University of Viçosa (registration 169/2009/DVT).

DMH Exposure and induction of the appearance of ACF

The animals that were assessed for the appearance of ACF were given intraperitoneal injection once a week of 20 mg/kg body weight DMH for 8 weeks. DMH was dissolved in 0.9% saline solution containing 1 mM EDTA and 10 mM sodium citrate, pH 8. Seven weeks after the last DMH administration, the animals were euthanized by CO₂ asphyxiation. The large intestine of the animals was removed from the cecum to the anus for counting ACF.

After removal, the large intestine was washed in phosphate-buffered saline (PBS) solution, opened along the mesenteric margin, placed in paraffin plates with the mucous facing the top of the plate, and fixed in Carson's formalin for 24 hours. Once set, the large intestine was measured and divided into three equal fragments, called proximal, middle, and distal, in relation to the cecum. Then, the fragments were stained with 0.1% methylene blue solution for 2 minutes. The assessment of ACF was performed using a BX-60[®] light microscope (Olympus, Tokyo, Japan) at 40x and 100x magnification. The ACF were counted across the mucosal surface of the large intestine, cecum to rectum, by two observers in a double-blind manner. ACF categorization was based on the number of aberrant crypts per focus: foci with fewer than or equal to three crypts (ACF \leq 3) and foci with more than three crypts (ACF $>$ 3)⁵.

Organ weights

After seven weeks from the last DMH application, when animals were euthanized, we weighed the liver, heart and kidney.

Quantification of Bifidobacterium spp., Clostridium spp. and total anaerobes

Bifidobacterium spp. was determined in gar MRS + NPML and total anaerobes were determined on Wi-

Ikkins-Chalgren Agar. The plated samples containing total anaerobes and *Bifidobacterium spp.* were incubated at 37 °C in an anaerobic chamber (Gas Pak System – BBL, Sparks, USA) for 48 hours and 72 hours, respectively. For counting *Clostridium spp.*, we used Reinforced Clostridial Medium (RCM) agar. The plated samples were incubated at 37°C in an anaerobic chamber (Gas Pak System – BBL, Sparks, USA) for 48 hours¹⁴. The analyses were conducted in duplicate and the results for counting *Bifidobacterium spp.* colonies, total anaerobes, and *Clostridium spp.* were expressed in Log₁₀ CFU/g.

RT-PCR: expression of cyclin-E, cyclin-D1, p16, p21, and p53 proteins

RNA was extracted after the maceration of the distal colon in an Eppendorf tube using TRIzol® (Invitrogen, CA, USA). We briefly homogenized 500 mL of the sample with 750 mL TRIzol® and the mixture was incubated at room temperature for 5 minutes. Then, we added 300 mL of chloroform, manually agitating the tubes for 15 minutes and incubating them at room temperature for 2 more minutes. The samples were centrifuged at 12,000 g for 15 minutes at 4°C. The transparent aqueous phase was subsequently separated and transferred to another tube. We then added 350 mL of isopropanol and the samples were incubated at room temperature for 10 minutes to promote the precipitation of RNA and then, they underwent centrifugation for 10 minutes at 4°C. The pellets were washed with 750 mL ethanol (75%), centrifuged for 10 minutes at 7,500 g and dried at room temperature for 30 minutes. When dried, pellets were resuspended in 20 mL diethylpyrocarbonate (DEPC)-treated water. Approximately 10 mg of each total RNA was used to synthesize the first cDNA strands, using primers for cyclin-D1, cyclin-E, p16, p21, and p53. After the extraction of total RNA from the samples, we performed the synthesis of the first cDNA strand for cyclin-D1, cyclin-E, p16, p21, and

p53 using the Superscript™ kit (Invitrogen®, New York, USA).

Quantification of mRNA for cyclin-D1, cyclin-E, p16, p21, and p53 by Real-time Polymerase chain reaction (RT-PCR)

The gene expression of cyclin-D1, cyclin-E, p16, p21, and p53 was evaluated in the distal colon. After 24 hours, the colon was cleared by centrifugation at 10,000 g for 2 minutes and subjected to RNA extraction and synthesis of complementary DNA, as previously described. The evaluation of gene expression was performed using the RT-PCR technique, in which the expression of these mRNAs was compared with the expression of the mRNA of b-actin, a constitutive gene. To this end, approximately 2 mg of total cDNA from the distal colon was used for the RT-PCR, in a 50 ml reaction that contained 0.45 nmol of sense and antisense primers suitable for each gene¹⁵, 20 U/ml of RNase inhibitor, and 12.5 ml of SYBR Green PCR Master Mix (Life Technologies do Brasil Ltda, São Paulo, SP). The cycling conditions were 95°C for 10 minutes followed by 40 cycles of 94°C for 1 minute, 56°C for 1 minute, and 72°C for 2 minutes. The quantification of transcriptional gene expression was performed using the Gene Amp® 5700 Sequence Detection System Version 1.3 software (Applied Biosystems, São Paulo, SP). The quantification of the transcriptional gene expression was performed using the Gene Amp® 5700 Sequence Detection System Version 1.3 software (Applied Biosystems, São Paulo, SP)¹⁵.

Statistical analysis

Results are expressed as measures of central trend means, and standard deviations (mean±SD). All tests were performed using the Statistical Analysing System 9.1 software USA. Comparisons between three or more independent groups were performed by variance analysis (ANOVA) for data with a normal distri-

The Quantification of the transcriptional gene expression was performed using the Gene Amp 5700 Sequence Detection System Version 1.3 software (Applied Biosystems)

<i>Genes</i>	<i>Primers</i>
Ciclina D1 (Rajkumar et al.; 2007)	Sense: 5'-CATCAAGTGTGACCCGGACTG-3' Antisense: 5'-CCTCCTCCTCAGTGGCCTTG-3'
Ciclina-E (Nakade, et al.; 2009)	Sense: 5'-AGACCCACACCAACAGCTTG-3' Antisense: 5'-TCATTCTGTCTCCTGCTCGC-3'
p16 (Muddhasani, et al.; 2008)	Sense: 5'- ATCTGGAGCAGCATGGAGTC-3' Antisense: 5'- TCGAATCTGCACCGTAGTTG-3'
p21 (Muddhasani, et al.; 2008)	Sense: 5'-GTCAGGCTGGTCTGCCTCCG-3' Antisense: 5'-CGGTCCCGTGGACAGTGAGCAG-3'
p53 (Hailfinger, et al.; 2007)	Sense: 5'- GGAGACATTTTCAGGCTTATGG -3' Antisense: 5'- AGAAGGGACAAAAGATGACAGG -3'

Table I

Number of aberrant crypt foci (ACF) with three or less crypts in the proximal, middle and distal colon and number of ACF with more than three crypts in the middle and distal colon of Balb/C mice exposed to DMH (20mg/Kg) for eight weeks

Treatments	Less or Equal to 3 ACF			More than 3 ACF	
	Proximal colon	Middle colon	Distal colon	Middle colon	Distal colon
C-	3.5±0.7 ^a	30.5±7.7 ^a	39.5±6.3 ^a	5.0±1.4 ^a	8.0±1.4 ^a
LINCA	2.5±0.8 ^a	5.5±0.7 ^b	8.5±0.7 ^b	0.0±0.0 ^b	0.0±0.0 ^b
LIN	0.0±0.0 ^b	0.0±0.0 ^c	0.0±0.0 ^c	0.0±0.0 ^b	0.0±0.0 ^b

Data are reported as mean±SD. (C-): AIN93M diet without fibers + DMH; (LINCA): AIN93M diet with defatted flaxseed meal + DMH; (LIN): AIN93M diet with Defatted flaxseed meal. ^{a,b,c}Different letters in the columns indicate statistical difference between groups (p<0.05), ANOVA one-way (Tukey's test).

bution. If there were statistical differences, we used the Tukey's multiple comparison tests and Lilliefors non-parametric test to detect groups that differed. The value (p) was fixed at 5% to obtain 95% of reliability in the comparisons for Tukey's test.

Results

ACF count in the proximal, middle, and distal colon

Analysis of the number of ACF with less than or equal to three crypts (ACF≤3) in the proximal, mid-

le, and distal colon showed that the group LIN, which did not receive DMH, not presented ACF in any of the segments of the intestine. The group LINCA presented fewer ACF in the middle and distal colon compared with control (C-) (p<0.05) (Table I). When we analyzed ACF with more than three crypts (ACF>3), the results showed that the groups LIN and LINCA did not present ACF in the middle and distal segments, in contrast to the control group (p<0.05) (Table I).

Organ weights

In relation to the weight of the organs, the groups LIN and LINCA showed higher values for the liver and kidney compared with control (C-), but heart weight did not differ between the groups (p<0.05) (Table II).

Bifidobacterium spp., *Clostridium spp.*, total anaerobes, and measurement of fecal pH

The groups LIN and LINCA showed increase in the amount of *Bifidobacterium spp.* compared with control (C-). However, there was no difference between groups in relation to the amount of *Clostridium spp.* or total anaerobes (p>0.05). Regarding the pH, the LINCA group showed a lower value compared to others groups (p<0.05) (Table III).

Table II

Heart, Liver and kidney weight of Balb/C mice exposed to DMH (20mg/kg for eight week)

Treatment	Organ weight/g		
	Heart	Liver	Kidney
C-	0.25±0.05 ^a	1.08±0.12 ^a	0.21±0.05 ^a
LINCA	0.20±0.03 ^a	1.65±0.21 ^b	0.50±0.06 ^b
LIN	0.24±0.04 ^a	1.58±0.13 ^b	0.50±0.06 ^b

Data are reported as mean±SD. (C-): AIN93M diet without fibers + DMH; (LINCA): AIN93M diet with defatted flaxseed meal + DMH; (LIN): AIN93M diet with Defatted flaxseed meal. ^{a,b}Different letters in the columns indicate statistical difference between groups (p<0.05), ANOVA one-way (Tukey's test).

Table III

Average count of bacteria Log₁₀ UFC/g and pH of feces in the colon for the different treatments

Treatment	Average count of bacteria			pH
	<i>Bifidobacterium spp.</i>	<i>Clostridium spp.</i>	Total anaerobes	
C-	7.67 ± 0.46 ^a	7.98 ± 0.41 ^a	8.97±0.72 ^a	7.75±0.24 ^a
LINCA	8.69 ± 0.54 ^b	8.91 ± 0.66 ^a	8.85±0.31 ^a	6.99±0.23 ^b
LIN	8.99 ± 0.63 ^b	8.49 ± 0.53 ^a	8.86±0.18 ^a	7.49±0.31 ^a

Data are reported as mean±SD. (C-): AIN93M diet without fibers + DMH; (LINCA): AIN93M diet with defatted flaxseed meal + DMH; (LIN): AIN93M diet with Defatted flaxseed meal; ^{a,b}Different letters in the columns indicate statistical difference between groups (p<0.05), ANOVA one-way (Tukey's test).

RT-PCR

When we analyzed the expression of the genes for p53, p21, and p16, the LINCA group showed significantly higher p53 compared with control ($p < 0.05$) (Fig. 1a). Regarding the expression of p21, although the group LINCA presented a higher expression compared with the other groups, there was no significant difference between them (Fig. 1b). The analysis of p16 showed no significant difference between groups LIN and LINCA ($p < 0.05$) (Fig. 1c). The quantification of cyclin-E/ β -actin showed that the group LINCA had increased mRNA expression compared with the other groups ($p < 0.05$) (Fig. 2a). Taking into account the expression of cyclin-D1/ β -actin, the group LINCA had a higher expression compared with the other groups but there was no significant difference between them ($p < 0.05$) (Fig. 2b).

Discussion

The present study investigated the preventive effect of defatted flaxseed meal on the appearance of ACF in mice treated with DMH. The experimental model used in this study was previously validated as a model of CRC⁴⁻⁶. Some studies have shown that the use of soluble fiber in the diet promotes the development of many bacteria including *Bifidobacterium spp.* that play a key role in maintaining the integrity of the intestinal microbiota, providing a composition of micro-organisms that produce a harmonious relationship with the host, promoting health maintenance or improvement of patients with gastrointestinal diseases¹⁶⁻¹⁹. Furthermore, it is known that soluble fibers, when fermented by *Bifidobacterium spp.*, act by inhibiting the growth of pathogenic bacteria including *Clostridium spp.* Another important action of this type of food in the diet is reduction of fecal pH, which provides bactericidal effects by stimulating the maintenance of normal microbiota¹⁶⁻¹⁹. In the present study, the groups that received flaxseed showed a significant increase in *Bifidobacterium spp.* when compared with control; however, no change was observed in the count of *Clostridium spp.* The data associated with the decrease in fecal pH suggest that flaxseed has a protective effect on the gastrointestinal tract against various kinds of diseases, since it helps to maintain normal intestinal flora and promotes a fall of fecal pH, increasing the antibacterial effect.

DMH and its metabolite azoxymethane (AOM) are pro-carcinogens that require metabolic activation to form reactive products that interact with DNA. This interaction promotes a number of systemic manifestations in target organs such as the liver, kidneys, and heart. The metabolism of these compounds involves multiple metabolizing enzymes in the liver that are formed by the action of CYP2E1 and then are transported to the colon via the bloodstream¹⁹. The possibility of DMH and AOM targeting the colonic mucosa is probably due to the relative stability of the hydroxylated metabolites

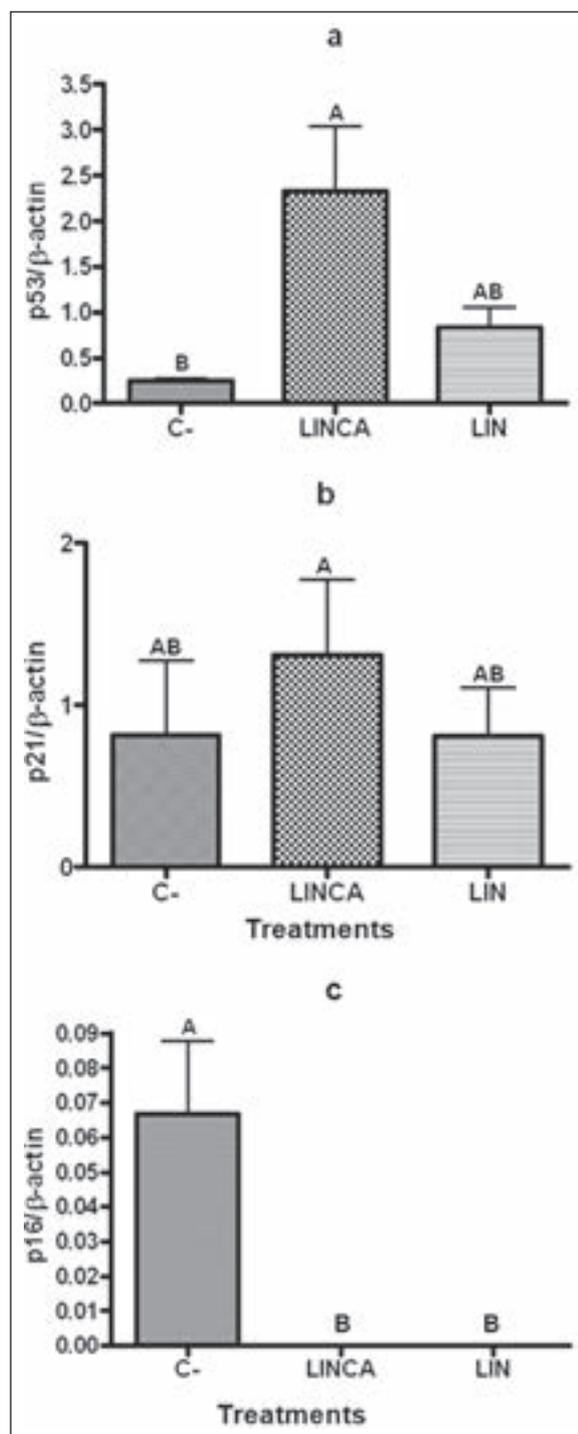


Fig. 1.—Quantification of p53, p21, and p16 mRNA in the distal colon of Balb/C mice exposed to 1,2-dimethylhydrazine (DMH) (20 mg/kg) for 8 weeks. ^{A,B}Different letters indicate statistical significance between the groups ($p < 0.05$), and groups with the same letter do not differ statistically (one-way ANOVA). Data are reported as mean \pm SD. (C-): AIN93M diet without fibers + DMH; (LINCA): AIN93M diet with defatted flaxseed meal + DMH; (LIN): AIN93M diet with defatted flaxseed meal.

of these compounds²⁰. Thus, a possible path for the investigation of the protective effect of defatted flaxseed meal may be the interference of food in any of the steps

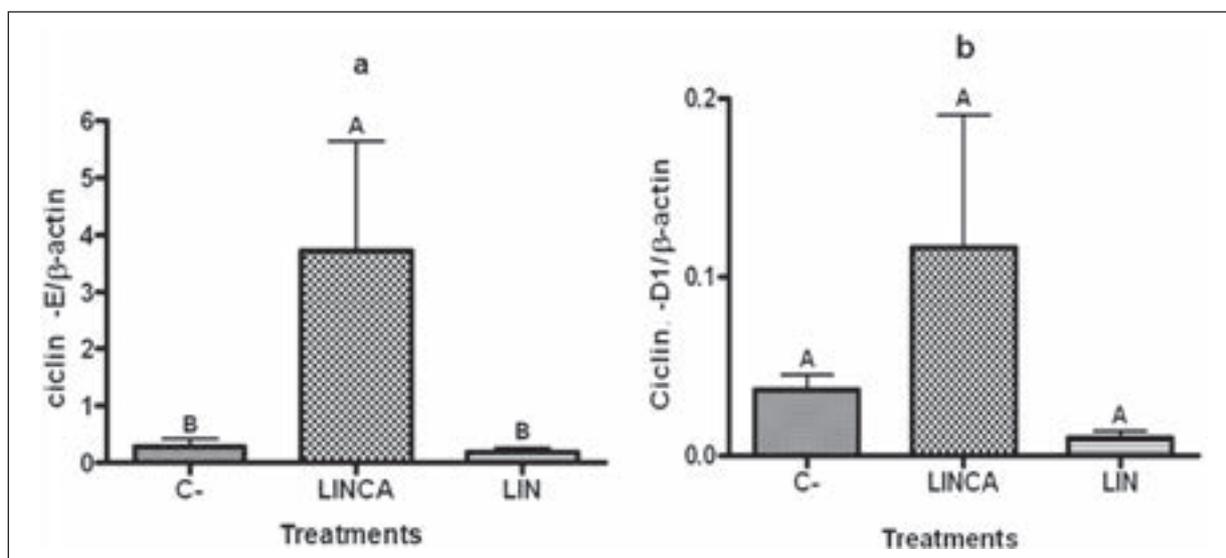


Fig. 2.—Quantification of cyclin-E/ β -actin and Cyclin-D1/ β -actin mRNA in the distal colon of Balb/C mice exposed to 1,2-dimethylhydrazine (DMH) (20 mg/kg) for 8 weeks. ^{A,B}Different letters indicate statistical significance between the groups ($p < 0.05$), and groups with the same letter do not differ statistically (one-way ANOVA). Data are reported as mean \pm SD. (C-): AIN93M diet without fibers + DMH; (LINCA): AIN93M diet with defatted flaxseed meal + DMH; (LIN): AIN93M diet with defatted flaxseed meal.

of the metabolic processes catalyzed by drug-metabolizing enzymes present in the liver, making them less active and also less toxic to other organs such as the heart, spleen, and kidney. In the present study, we observed a decrease in liver weight and kidney in the control group, suggesting a possible change in homeostasis caused by DMH in these organs. These results suggest that the defatted flaxseed meal may have protected the liver and kidney from the deleterious effects of DMH.

Some studies have shown the beneficial effects of soluble fiber in the prevention of the onset of ACF through activation of the immune system²¹⁻²⁴. p53, p21, and p16 are negative regulators of cell growth, since they act as a tumor suppressor by inducing the expression of gene products responsible for the interruption of the processes of cell growth^{25,26}. It has been observed that these markers act together in the process of tumor development and that p53 is responsible for the regulation of p21, since p53 mutations can lead to loss of function of p21, causing uncontrolled cell cycle progression, and can be a fundamental mechanism in the progression of CRC²⁷. In a study of 188 patients with adenocarcinoma, it was observed that loss of p21 expression was related to lower p53 protein expression²⁸. In addition to these markers, a strong association between the expression of p16 and inhibition of the cell cycle has been observed, being directly connected to the evolution of better prognosis of patients²⁹. In this study, the group treated with linseed bran showed higher p53 expression and lower number of crypt foci compared with control. These data suggest that flaxseed protected the colonic mucosa from tumor development due to increased expression of p53, an important tumor suppressor. In relation to p21, the LINCA group also showed an increase in expression, but not enough to differentiate itself from the other

groups. These data suggest that there may have been an influence of p53 on p21, however, other mechanisms may also have acted to protect the middle and distal colon of the mice to prevent the development of tumors, since there were no differences between the groups regarding p21. In addition to the markers described above, there are other proteins associated with cell cycle inhibition and, consequently, with the development of better prognosis. Among them are cyclins D and E, which act on the influence of p21 that inhibits cyclin-dependent kinases (cdk2 and cdk4) required for cell cycle progression²⁹. In the present study, we found that p21 did not influence the decrease in gene expression of cyclin E and cyclin D in the LINCA group. The increase in cyclin D in the LINCA group in the present study may be related to renovation of the mucosal epithelium of the distal colon and not directly to the increase in ACF, once p53 could trigger a reduction of cells in ACF, once that for LINCA treatment was not observed FCA > 3.

In colon cancer, studies have evaluated the relationship between the expression of p16 and its association with the inhibition of cell cycle and the evolution of better prognosis of patients^{30,31}. Strong cytoplasmic immunostaining of p16 indicates a better prognosis, whereas strong immunostaining of Cdk4 indicates poor prognosis of tumors. These results indicate that the correlation between the expression of p16 and Cdk4 may play an important role in the mechanism of inhibition of retinoblastoma by p16. Thus, the expression of p16 and Cdk4 can play a key role in the development of CRC, becoming new prognostic markers in colorectal carcinoma³¹. In the present study, no differences in the expression of p16 and cyclin D1 mRNA, therefore,

was not possible to suggest that these proteins might be having an effect in inhibiting the onset of ACF.

Conclusion

The present study showed that defatted flaxseed meal exerted a protective effect on the appearance of ACF in mice treated with DMH. The results indicated a possible reduction of morphological and functional damage to the colorectal parenchyma by defatted flaxseed meal, which may be associated principally with increased expression of the p53 gene and a greater number of *Bifidobacterium spp.* associated with reduced fecal pH. Further evaluations are essential to identify the active components in defatted flaxseed meal and fully clarify its mechanism of action, which may be associated with a high potential for the prevention and treatment of colorectal cancer.

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