RESEARCH COMMUNICATION

Cancer Chemopreventive Potential of the Egyptian Flaxseed Oil in a Rat Colon Carcinogenesis Bioassay - Implications for its Mechanism of Action

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Abstract

The possible chemopreventive effects of natural Egyptian flaxseed oil on preneoplasia and cancer formation were investigated in a rat medium-term colon carcinogenesis bioassay. Male Wistar rats were divided into 6 groups. Groups 1, 3 and 5 were initiated by 1,2-dimethylhydrazine (DMH) 20 mg/kg body weight s.c. 8 times, twice a week to initiate colon carcinogenesis. Groups 1 and 3 received 20% or 5% flaxseed oil respectively in diet in post initiation stage until the end. Groups 2 and 4 served as a flaxseeds dose corresponding controls without carcinogen initiation, while rats in group 6 served as negative controls. Distribution and total numbers of aberrant crypt foci (ACF), putative preneoplastic lesions, particularly those with ≥4 aberrant crypts (ACs), and the numbers and sizes of colon tumors (adenoma and carcinoma) were significantly decreased by both treatment doses of flaxseeds as compared to group 5. Histochemical investigation revealed that the numbers of mucus-secreting cells in the colonic mucosa were reduced gradually during progression of colon carcinogenesis. Intriguingly, flaxseed oil caused the numbers and integrity of the mucus-secreting cells to retain close to normal levels and in a dose dependent manner. Moreover, the hematological parameters were almost constant between the groups particularly at the dose of 5% as compared to groups 5 and 6. PCNA-labeled indexes (PCNA-LI) in the DMH-initiated colonic mucosa were found to be decreased by both doses of flaxseeds administration. In conclusion, the present study showed that the post initiation dietary administration of flaxseeds oil suppressed DMH-induced colon carcinogenesis in rats without significant side effects. The mechanism is likely to be through its inhibitory effects on early cellular proliferation and modulation of mucin secretion properties in the initiated colonic mucosa.

Keywords: Egyptian flaxseed oil - chemoprevention - rat colon model - mucin production

Asian Pacific J Cancer Prev, **12**, 2385-2392

Introduction

The search for compounds that prevent cancer has intensified with the mounting evidence that many types of cancer are caused or triggered by factors relating to lifestyle and nutrition. Recent findings support a growing body of evidence that flaxseeds, or its extracted oil exert anti-carcinogenic effects in some in vitro and in vivo experiments, and that flaxseeds oil and related extracts also play an important dietary role in various biological activities in the body (Basch, 2007). Flax is an erect annual herb of the family of Linnaceae plants growing in many countries, especially those ringing the Mediterranean Sea. Flax seeds are the richest plant source of ω-3 fatty acid (α-linolenic acid) and the phytohormone lignans. The Egyptian flaxseeds are reputed by its better flavor and color other than other types. It is also an essential source of high-quality protein and dietary fiber (ZUK, et al., 2011).

Although ω-3 fatty acids have been associated with improved cardiovascular outcomes, ω-3 fats are used by the body to produce series 1 and 3 prostaglandins, which are anti-inflammatory hormone-like molecules, in contrast to the series 2 prostaglandins, which are pro-inflammatory molecules produced from other fats, notably the ω-6 fats, which are found in high amounts in animal fats and corn oil (de Giada, 2010). Moreover, flax lignans which belongs to the phytoestrogens are metabolised after ingestion into enterolignans that may offer a protection against the onset and development of hormono-dependant cancers (Lamblin et al., 2008). Because of the beneficial physiological effects of its components, this seed oil can contribute to the reduction of several diseases such as diabetes mellitus, arteriosclerosis and cancer (de Giada, 2010).

Colon carcinogenesis is a multistep process caused by a series of genetic or subsequent epigenetic alterations that are indispensable to different stages from initiation to promotion and progression of cancer development (Fearon and Vogelstein, 1990). ACF are now frequently used as effective surrogate biomarkers for experimentally detection of cancer promoters or chemopreventive agents against colorectal cancers (Fernandes et al., 2011). Recently, evidence has also shown that ACF can be effectively

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employed in chemopreventive studies in humans (Patlolla et al., 2011). ACF appear at an early period of colorectal carcinogenesis and in the tumor- bearing rodents after treatment with chemical carcinogens and in patients with familial adenomatous polyposis (FAP) and other bowel tumors (Roncucci et al., 1991). Increased ACF size and frequency is closely related to tumor promotion (Tang et al., 1996). Their dysplastic appearance, increased cell proliferation and high frequency of K-ras and p53 mutation (Losi et al., 1996) point to a preneoplastic role of ACF during colorectal carcinogenesis in human and in rodents (O'Leary et al., 2011).

On the genetic level ACF have been shown to be associated with C-foc (Zahashi et al., 1993) and ras mutations in rats (Pera and Bird, 1992) and humans (Vivona et al., 1993). Also ACF have been demonstrated to be useful biomarkers for screening chemopreventive agents against colon cancer (Corpet nd Tache 2002). ACF also express mutations in the APC gene and ras oncogene that are involved in colon cancer development (Vivona, et al., 1993).The pathogenesis of colorectal cancer begins with increased proliferation or decreased apoptosis of epithelial cells followed by adenoma formation, dysplasia, invasion, and ultimately metastasis (Kinzler and Vogelstein, 1996). Alterations of tumor suppressor genes and oncogenes have been identified at various stages of tumorigenesis (Paulsen, 2000).

Genotoxic carcinogenic substances in general, enhance cellular proliferation in their target tissue with a strong target organ specification. For example, DMH enhances cellular proliferation in the colon but not in other organs of the experimental animals (Ohno et al., 2001). In the colon, increased numbers of cycling cells, particularly cells in the G1-S phase of the cell cycle, increased mitosis, both leading to expansion of the cell proliferating zone and increased crypt heights, as well as deregulation of apoptosis are considered risk factors for tumor development (El-Najjar et al., 2007). The proliferating cell nuclear-antigen (PCNA) is an auxiliary protein for DNA-polymerase delta (δ) present in the S-phase of the cell cycle. It is expressed in the genomic DNA during the process of cellular division (G1 phase) and growth (S-phase) (Shivji et al., 1992). PCNA has been widely used for evaluating cell proliferative activity in animal models used to test carcinogenesis or cancer prevention (Corpet and Tache, 2002).

The mucus layer of the colon and most parts of the alimentary canal, which is also known as the suparmucosal defense barrier, is composed of large mucin molecules. These molecules are synthesized in a site specific fashion by the mucosa of the gastrointestinal tract. Mucins are heavily glycosylated molecules that consist of polypeptide backbone and o-linked oligosaccharide chains, which confer the characteristic viscosity necessary for the formation of mucus gels (Corfield and Warren, 1996). These gels serve to lubricate and protect the mucosal surfaces of the respiratory, gastrointestinal and urogenital tracts. Glycoproteins in mucin have many biological functions, including physicochemical protection from acids, enzymes, toxins and mutagens, adhesion modulation, signal transduction, and regulation of cell growth (Moniaux et al., 2001). Each type of mucin has a characteristic tissue distribution (Reis, et al., 1997). The colon normally expresses the mucins MUC1, MUC2, MUC3 and MUC4 (Allen et al., 1998), MUC2 is particularly the main and most predominantly secreted mucin glycoprotein, and is confined to mucus cells. The MUC2 expression was found to be decrease in some poorly differentiated human colorectal tumors (Sylvester et al., 2001), but little is known about their molecular expression in chemically-induced rat colon carcinogenesis (Bara et al., 2003). Until now, about 20 mucin genes are known to work secreting different mucin gels in the digestive tract of human (Kim et al., 2011).

In the present study, the possible inhibitory effects of the flaxseeds oil on DMH-induced rat colon carcinogenesis was studied using colon tumors (adenoma and carcinoma) as end-points of colon carcinogenesis, in addition to the distribution and sizes of ACF which are early biomarkers of colon carcinogenesis. The possibility of toxic side effects of flaxseeds oil was also investigated using quantitative analysis for biochemical parameters of the blood components such as the serum levels of total proteins, albumin, triglycerides (TG) and cholesterol. Animals' growth rates and histopathology of different organs and tissues were also extensively studied. An approach to the mechanism of the anticancer effect of the flaxseeds oil was estimated depending on its effect on early cellular proliferation and on the mucus-secretion status in the colonic mucosa.

Materials and Methods

Animals:

Male 6-week old Wistar rats were obtained from Theodor Bilhariz Institute breeding facility, Warrak AL-Hadar, Cairo. The weight of rats ranged from 79 to 93gms at arrival. Prior the experiment, animals were housed for one week acclimation period, 4-5 per cage in plastic cages with wood chips for bedding in conventional animal house. The animal room was adjusted with temperature of 21±3ºC and 12h light-dark cycle. The room humidity was around 50±5. Diet and water were available ad libitium, and body weights, food consumption and water intakes were measured weekly during the course of the experiment.

Chemicals and diet:

DMH, 1, 2-Dimethylhydrazine dihydrochloride (purity 99%) of the chemical formula (C2H8N2.2HCL) was obtained from Fluka Company (Sigma-Aldrich, st. Louis. USA). Animals were given subcutaneous (s.c.) injections of DMH dissolved in normal saline solution (0.9%), 20 mg /kg body weight. Animals were fed standard rabbit diet obtained from B.M. Company for International Trade and Development, Fouda Factory for animal's foods, EL-Baramoon, EL-Mansoura, Farskor road, Egypt. This basal diet was composed (v/v) percentage of dried grass, soy bean, wheat bran, corn, mixed vitamins, minerals (salts), bones powder, bean straw, D.L. methane. These contents present with different percentage were as follows: Crude protein: not less than 18%; crude lipid, not less than 2.6%;

crude fiber, not more than 11.43% and the ingestion energy was not more than 284.83 k cal/100g.

Crude extracted flaxseeds oil was purchased fresh from Awara Co., Tanta, Egypt. The oil was stored shaded from light in dark glass bottles at temperature between 15ºC and 20ºC. The chemical composition of flaxseeds oil is: 30-40 % fixed oil includes linoleic, linolenic, oleic, palmitic and stearic acids, mucilage (6%), protein (25%), the cyanogenic glycoside linamarine, bitter, and also contains vitamins A, B, D and E, minerals and amino acids (Campbell, 2003). For the diets containing flaxseeds oil, the oil was mixed in (v/v) ratio to the basal diet at doses of 5% and 20% respectively. The diet was prepared and provided fresh every two-three days.

Experimental design (Figure 1):

After acclimation period, the rats were separated into 6 groups. Groups 1, 3 and 5 including 8 male rats were given 8 subcutaneous (s.c.) injections of DMH (20mg/kg body. weight) twice a week, for 4 weeks. Groups 1, 2 and 6 including 5 male rats each, were injected with 0.9% normal saline solution as vehicle at the same dose and time of the DMH administration in groups 1, 3 and 5. Two days after the last injection, the food containing flaxseeds oil was prepared as mentioned above and introduced fresh to the rats every two days. Group 1 and group 2 were fed diet containing 20% flaxseeds oil. Group 3 and group 4 were fed diet containing 5% flaxseeds oil and groups 5, 6 were fed the basal diet without flaxseeds oil. All animals were sacrificed after 32 weeks of the start of the experiment.

Necropsy and tissue preparation:

Animals were killed in the desired day under excess chloroform anesthesia. The abdomen was opened, and the entire colon was removed. The colons were opened longitudinally, flushed clean with 0.9% normal saline solution and examined for the presence of tumors. Other body organs were observed and checked for any abnormalities or lesions. All grossly visible colonic lesions were sketched and marked on charts to record their locations and to facilitate precise histological investigation. To evaluate tumor volume (v), the long (L) and short (w) dimension (mm) of each tumor mass (length and width) were measured with calipers and calculated according to the equation: v= (L X W2/2) (Shen, et al., 2003). Then tumors were fixed in 10% phosphate buffered formalin for at least 48 hours and processed for histopathological examination. All colons were blotted between two filter papers and fixed in the same fixative for at least 48 hours until ACF count assay. Vital organs such as the livers, kidneys and spleens were excised, weighted to calculate absolute and relative organ weights (organ weight / body weight x 100), and sliced then preserved in the same fixative until prepared for routine histopathological examination for further estimation of any incidental toxicity or histopathological changes that might have occurred due to outer unfavorable factors like infection, that might influence the numbers and progression of the preneoplastic lesions or tumors.

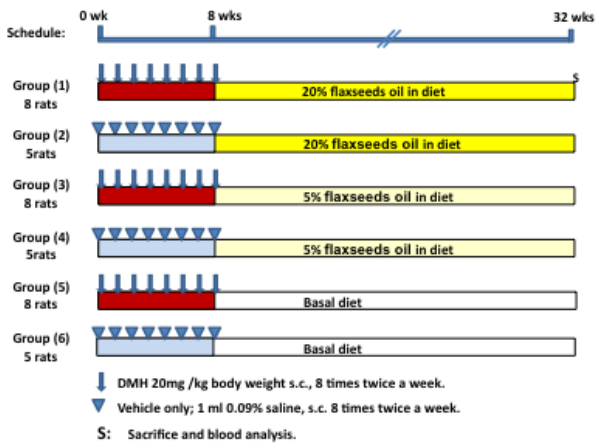

Figure 1. Experimental Design

ACF count assay:

After fixation in 10% phosphate buffered formalin, colons were stained with 0.02% methylene blue in distilled water for 2-5 minute. After staining they were divided into 3 equal parts namely; the caecal ascending part (the proximal colon), middle part (the intermediate, or mid colon), and descending part (the distal colon). Samples were examined for the presence of ACF by compound microscope at x10 and x20 magnifications using the following criteria for identification: increased size compared to normal crypts, enlarged pericryptal zone, slight elevation above the surrounding mucosa, and more elongated shape of the luminal opening. The number of aberrant crypts in each focus was counted and divided by the total numbers of aberrant crypts to evaluate the crypt multiplicity. Scoring of ACF was performed in each part separately to detect variations in induction (site-specificity). ACF that showed variations in its multiplicity, such as foci containing 1 crypt (1AC), foci with 2 or 3 crypts (2ACs and 3ACs), or larger foci with- or more than 4 crypts (≥4ACs), were counted and separated in categories. To calculate the ACF density in the colonic mucosa/cm^2 (ACF/cm^2), the mean lengths and widths of each colonic segment were measured to determine mean area of the colonic mucosa.

Histopathological evaluation:

Directly after ACF counting, samples collected from the proximal, intermediate and distal colons; were dehydrated and embedded in paraffin wax, sectioned at 4-5 μm in thickness and stained routinely with heamatoxylin and eosin as well as Alcian blue for histopathological and histochemical examinations respectively.

Alcian blue histochemistry and mucus cell counts:

Alcian blue (pH 2.5) is considered the most specific dye available for mucopolysaccharides and mucins using1 gm Alcian blue and 100 ml of 3 % acetic acid. Sections of proximal, mid and distal colons of all groups were rinsed in 3% acetic acid for 5 min. All sections were stained for 30 min in Alcian blue solution. Then sections were rinsed for 10 min in 3% acetic acid again to prevent non specific staining. After that, sections were washed in distilled water, dehydrated through alcohol series, cleared in xylene and mounted in Canada balsam. To determine whether DMH caused morphological changes in the mucus cell

(mucus cell) numbers and integrity, Alcian blue-positive cells were quantified for each category of DMH-treated mucosa and control mucosa (non-treated). The mucus cell numbers were counted in the colons of 5 rats from each group. The percentage of the total numbers of mucus cells in at least 1000 cells of mixed epithelial and mucus cells in random fields was calculated under light microscope at x400.

Proliferating cell nuclear antigen (PCNA) immunohistochemistry:

Immunohistochemical staining was performed according to the avidin-biotin complx (ABC) method. Briefly, tissue sections were deparaffinized with xylene, hydrated through a graded ethanol series and incubated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The sections were then incubated with 10% normal horse serum at room temperature for 30 min to block background staining and then incubated with Anti-PCNA antibody (PC-10, IgG2a; Dako, USA) diluted 1:500 in tris(hydroxymethyl) aminomethane-buffered saline (pH; 7.1) over night. The sections were then exposed to biotinylated horse anti-mouse IgG (Vector Labs. Inc. Burlinghame, CA, USA) for 30 min. Finally peroxidase activity in the colonic cell nuclei was visualized by treatment with 0.02% diaminobenzidine. Nuclei were counterstained with hematoxilin. To evaluate the PCNA-labeling index (cellular proliferation index), numbers of positively stained nuclei in all apparently complete crypts found in at least 10 sections from each colon were counted and divided by the total number of nuclei of each crypt X 100 to generate PCNA-LI (%).

Analysis of blood:

Blood was collected at autopsy using 5 ml syringes from the abdominal aorta from all rats and immediately centrifuged cold at 3000 rpm for 15 min. Serum was collected and preserved frozen at -30ºC before colorimetric determination and measurement of total protein and albumin according to Henry (1964), Total cholesterol and triglycerides (TG) concentrations in the serum were determined enzymatically using commercially available reagent kits (assay kits for the TDX system; Abbott Laboratory, Irving, TX, USA) as previously described by Bucolo and David (1973) and Allain et al., (1974). Total-cholesterol levels were calculated using the Friedewald equation (Wasan et al., 2001).

Statistical analysis:

The data obtained in the present work were represented in tables as mean ± standard deviation (S.D.). Significance of differences between animal groups mean values of body weight, tumors incidence and multiplicities, ACF numbers, blood biochemistry and immunohistochemistry data were calculated according to Student's t-test.

Results

Average body, liver and kidney weights:

Animals received flax seed showed increased body

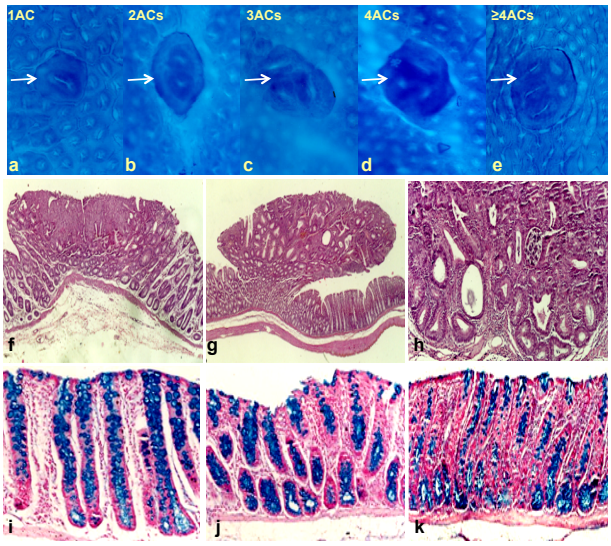

Figure 2. Histopathological and Histochemical Findings. (a-e): Methylene blue-stained whole mount colons showing foci with 1, 2, 3, 4 and ≥4 aberrant crypts (Acs). Methylene blue. x400; (f-g): Examples of tumors detected in DMH-treated colons: (f): dysplastic polyp, (g): adenoma, (h): adenocarcinoma. HE. x100; (i-k): PAS reaction results: (i): PAS reaction in colonic mucosa treated by DMH+20% flaxseeds oil, (j): PAS reaction in colonic mucosa treated by DMH+5% flaxseeds oil, (k): PAS reaction in colonic mucosa treated by DMH only showing reduced staining intensity and less numbers of mucus cells as compared to (I&J). PAS technique x200

weights which was significant only in the groups received 20% flax seed in diet as compared to the rats in groups 5 and 6. Regarding average and absolute liver and kidney weights, only rats of group1 (DMH+20% flax oil) and group 3 (DMH+5% flaxseeds oil) had significant increase in the absolute liver weights ($p<0.005$) as compared to the control rats of group 5 (DMH only). However, the relative liver weights were not different among the groups. The relative kidney weights were also similar among the groups. Absolute right and left kidney weights were significantly increased in groups 1, 2, 3 and 4 ($p<0.01$) as compared to group 5.

Total numbers and multiplicities of ACF in the colons:

All animals treated with DMH showed 100% incidence of ACF, in contrast to the complete lack of such lesions in the corresponding control rats without DMH treatment (Figure 2a-e). In group 5 (DMH only), DMH induced a total of 156.9±76.4 ACF per rat, while the dietary administration of flaxseeds oil at the dose of 20% (group 1) significantly ($p< 0.009$) reduced the total numbers into 53.2±26 ACF per rat, and the dose of 5% (group 3) reduced

Table 1. Incidence and Multiplicity of Colon Tumors (Adenomas + Carcinomas)

Group/Treatment		No.	Incidence (%)[a]	Multiplicity[b]
1	DMH+20% Fx	8	100	0.25±0.13*
3	DMH+5% Fx only	8	75	0.18±0.19*
5	DMH only	8	100	0.48±0.24
6	0.9% Saline	5	0	0

No, Number of rats per group; Fx, Flaxseed oil; [a]No. of rats bearing colon tumors per group (%); [b]No. of tumors per tumor bearing rat; Means ± S.D.

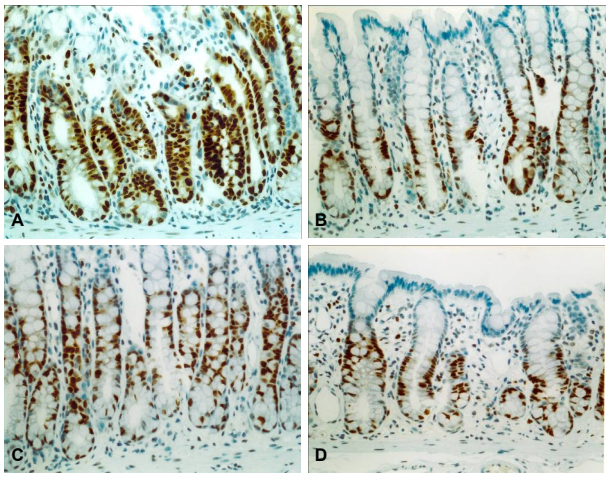

Figure 3. PCNA Immunohistochemistry. A) Colonic mucosa from a rat in group 5 administered DMH only without treatment. Note the high numbers of PCNA-labeled cells found in the basal and the upper parts of the colonic crypts. x400; (B-C): PCNA-labeled cells in the colonic mucosa of rats from groups 1 and 3 treated with 20% or 5% dietary flaxseeds respectively after DMH administration. Note relatively lower numbers of PCNA-labeled cells as compared to (A); (D): Colonic mucosa of a rat from group 6 administered 0.09% saline only. Note lower colonic crypt heights and decreased numbers of PCNA-labeled cells as compared to A

the numbers significantly ($p<0.025$) to 63.8±20.5 ACF per rat as compared to control group 5. The numbers of foci containing 1AC and these with 2ACs were decreased significantly in group 1 ($p<0.03$) and group 3 ($p<0.01$) as compared with the data of group 5. Also foci containing ≥4 crypts were significantly decreased in group 1 at $p<0.05$ as compared to group 5.

Multiplicity, incidence and tumor volumes:

Most tumors developed in the colon were mainly in its middle and distal areas, while none was found in the proximal segment. The tumors were sessile or pedunculated, and histologically diagnosed mostly as adenomatous polyps, dysplastic polyps, adenomas or adenocarcinomas (Figure 2f-h). Data for the average multiplicities (numbers of tumors/rat) and incidences (percentage number of rats bearing tumors per group) are summarized in Table 1. The multiplicities of tumors were significantly inhibited by both doses of flaxseeds oil administration ($p<0.03$ and $p<0.19$ respectively), while the incidence of the colon tumors (adenomas and carcinomas) in the flaxseeds oil treated group were lower in rats of group 3 (5% flaxseed oil) than those in the DMH-only treated (group 5), albeit without statistical significance. The average tumor volumes were decreased in both flaxseeds oil-treated groups by about 20% and 60 % respectively as compared to tumors found in group 5 (DMH only) with significant values obtained in the tumor volumes of group 3.

Alcian blue histochemistry and mucus cell counts:

Figure 2i-k shows the Alcian blue-stained acid glycoprotein (mucin) in the mucus-secreting cells in normally appearing mucosa (without tumors or ACF) in both non-treated control and DMH-treated rats. In non-treated control rats the Alcian blue staining was strong and intense in all colonic areas (proximal, middle, and distal colons). On the other hand, the mucosa of DMH-treated rats showed faint and weak Alcian blue staining. The mucosa of DMH-treated rats which had dietary flaxseeds oil showed an increase in the mucus cell staining ability as well as obvious increased mucus cell sizes and numbers when compared to those found in the mucosa of DMH only -treated rats of group 5. The average mucus cell numbers in DMH+20% flaxseeds oil mucosa (group 1), DMH only-treated mucosa (group 5) and control (saline) treated mucosa (group 6) were 490, 550, 210 and 660/1000 cells. Interestingly, the mucus cells were significantly fewer in numbers in the DMH only-treated mucosa as compared to non-treated (saline) control mucosa ($p<0.02$). Treatment with dietary flaxseeds oil in group 1 significantly increased the numbers of mucus cells as compared to DMH only-treated group at ($p<0.05$), and slightly increased the mucus cells numbers to levels close to control mucosa in group 6 without significant differences.

PCNA-LI (%) in the colonic mucosa:

Generally, the PCNA-LI were significantly higher in the DMH-only treated colons than the other groups ($p< 0.05$). In control non-treated animals, the PCNA-positive nuclei were mainly restricted in the lower one third of the colonic crypts of non-treated animals. DMH treatment generally increased the numbers of positive cells and extended the length of the proliferating zone in the colonic crypts to the middle and the upper thirds (Figure 3). However, treatment with flaxseed oil has lowered significantly the PCNA-LI the colonic mucosa of rats in groups 1 and 3.

Blood biochemistry:

Among serum concentration levels of total protein, albumin, cholesterol and triglycerides, values for both total protein and cholesterol of group 1 (DMH+20% flaxseeds oil) were significantly higher than normal control levels of group 6 (saline) at ($p< 0.01$ and $p<0.009$ respectively) (see Table 2). However, these levels were not significantly

Table 2. Blood Concentration Levels of Total Protein, Albumin, Cholesterol and Triglycerides (Experiment II)

Group	Treatment	No.	Total Protein[a]	Albumin[a]	Cholesterol[a]	Triglycerides[a]
1	DMH+ 20 % Fx	8	8.37±1.14*	7.02±3.90	151.60±41.6*	87.70±15.9
2	20 % Fx only	5	7.39±2.04	4.82±0.09	138.02±36.1	79.80±10.1
3	DMH+ 5 % Fx	8	6.42±0.72	5.28±0.54	092.96±30.2	80.51±19.5
4	5 % Fx only	5	7.29±0.94	4.49±0.27	097.90±53.0	41.50±12.5
5	DMH only	8	6.60±1.50	4.42±0.42	095.04±28.7	81.33±29.0
6	0.09% Saline only	5	5.52±1.83	5.06±0.90	076.13±13.0	65.53±8.62

No, Number of rats tested per group; [a]Values are means ± S.D; Fx, Flax seed oil; *Significant vs. group 5

different when compared with the DMH control levels of group 5. Other parameters were comparable among the groups.

Discussion

Colon cancer incidence is high in Western countries, some parts of Asia and Africa. The incidence is increasing in the developing countries including the Middle East and North Africa due to the increasing consumption of high-fat diet, less exercise, environmental pollution and Westernization of life style (Ferlay et al., 2010). Therefore, researchers work to develop new tools for better understanding the etiology and preventing colon cancer on the pathological and molecular levels. This study was designed to investigate the possible chemopreventive effect of natural oil from Egyptian flaxseeds on colon cancer in a rat medium-term (32-week) bioassay.

An attempt was made to clarify some possible mechanisms by which flaxseeds oil may exert its anticarcinogenic potential. In this study we used an experimental model of the colon carcinogenesis induced by DMH in rats with putative preneoplastic aberrant crypt foci (ACF) as preneoplastic marker lesions and colon tumors as end point. In the present experiment, the total numbers of ACF particularly those with higher crypt multiplicity consisting of ≥4ACs were significantly suppressed by flaxseeds oil administration in both doses. Pretlow et al., (1994), showed that aberrant crypts with large tumor multiplicity (≥4ACs) correlate with tumor incidence other than aberrant crypts with smaller crypt multiplicity. Also colons possessing higher numbers of ACF with higher crypt multiplicity have a higher risk of developing colon cancers (Seike et al., 2006). Therefore, the present findings of the reduction of total numbers and large ACF (≥4 crypts /focus) by flaxseeds oil might point to the further effect on tumor yield. Reduction of ACF number and multiplicity, as well as the relation of cancer chemoprevention in general to the initiation phases of carcinogenesis has been discussed widely (Sengottuvelan et al., 2007). In line with our results, a previous study of Serraino and Thompson (1992) showed a significant reduction (41-58%) in the numbers of azoxymethane (AOM)-induced AC and ACF in F344 rats with flax seeds intake (not the oil), while the study of (Jenab and Thompson, 1996) showed significant reductions primarily in AC multiplicity only, as opposed to the numbers of AC and ACF by dietary flax seeds.

In the present study we assayed the cellular proliferative status by the PCNA-positive labeled cell index (PCNA-LI) in colonic mucosa. It is well documented that the increase in this parameter positively correlates with developing colon cancer in the initiation phase (Fang et al., 2007) while other genetic factors take place for tumor formation. Our results demonstrated modulating effects of flaxseeds oil on DMH-induced cellular proliferation of rats' colonic epithelia in a dose-dependent manner. The DMH- only treated rats had increased incidence of PCNA-labeled G0-S phase-cycling colonic epithelial cells extended to the upper third of the crypts. The presence of DNA-synthesized cell in this location following carcinogen administration is indicative of greater damage to the mucosa and the development of the first proliferative defect recognized in a preneoplastic mucosa (Shpitz et al., 1998). With the accumulation of additional defects, the mucosa may proceed to develop an adenoma. The present study indicated that the inhibitory effects of flaxseeds oil on cellular proliferation might have played a role in the inhibition of total numbers of ACF and subsequently on the yield of the colonic tumors including adenomas and carcinomas and caused a reduction in their volumes. This reduction of the tumor volumes indicates that flaxseeds oil in the present study affected not only the initiation but also the progression of colon tumors.

Previously, it has been reported that the dietary fatty acid composition can influence the initiation, promotion and progression stages of experimental carcinogenesis in rats and mice (Augenlicht et al., 1999). Flaxseed oil is one of the richest dietary sources of α-linolenic acid 47.4% (ALA), which is a ω-3 polyunsaturated fatty acid (ω-3 PUFA) (cis-c18: 3 ω-3) (Cunnane, 1995). The consumption of purified ω-3 PUFA reduces colon cancer risk in humans and experimental animal models (Caygill et al., 1996). Recently consumption of oils rich in ω-3 polyunsaturated fatty acids was shown to attenuate the activity of a number of biochemical pathways implicated in the aberrant regulation of proliferation or cell death during tumorigenesis (Hong, et al., 2005). α-linolenic acid is converted to eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) after enzymatic de-saturation and elongation in the liver. ω-3 PUFAs have been reported to suppress rectal cell proliferation in humans (Rao et al., 1995). Flax is also rich in lignans providing 75 - 800 times more lignans than most other plant sources. Lignans have numerous biological properties particularly a potent antioxidant activity (Hu et al., 2007). Flax lignans are known to reduce cancer risk by preventing pre-cancerous cellular changes and by reducing angiogenesis and metastasis (Adolphe et al., 2010). Thus there is a possible involvement of flax lignans in addition to α-linolenic acid in the present study as a precursor of the inhibitory effects of flaxseeds oil on rat colon carcinogenesis. Importantly, this needs further subsequent studies.

Interestingly, the histochemical findings presented here showed a significant and a dose-dependent increase in the mucus cell numbers in the groups treated with flaxseeds oil after DMH administration as compared to the data of DMH-only treated group. The mucus cell numbers in flaxseeds oil-DMH treated groups were increased into numbers relatively near to the normal curve. Many studies have demonstrated that analysis of the epithelial mucins at the histochemical, biochemical, and genetic levels have the potential to predict and monitor the progression of colon cancer (Corfield et al., 2000). Altered mucin expression has long been associated with the pathology of epithelial diseases such as inflammatory bowel disease and respiratory diseases including cystic fibrosis in humans (Harris and Reid, 1997). Bara et al. (2003) have shown depletion in mucin secretion in gastric mucosa and colonic ACF of rats during progression of cancer in both organs. Our previous study on the ultrastructure of ACF

(Salim, 2005) described that the progression of ACF into adenoma is accompanied with a clear depletion in the numbers and integrity of mucus cells in both normally appearing mucosa, ACF and in tumors. In addition, Salim (2006) showed that the mRNA expression of two mucin producing genes; MUC2 and MUC5ac was significantly decreased during the progression of colon cancer, and indicated that this finding could be used as a future biomarker for colon carcinogenesis.

On the other hand, the analysis of the hematological biochemical parameters such as total proteins, albumin, TG and cholesterol levels in the present experiment showed no significant differences between most of the groups treated or untreated with flaxseed oil except that cholesterol and TG levels were increased in group 1 as compared to the levels of group 5. In a few laboratory and human studies, flaxseeds and its oil are reported to lower blood cholesterol levels. Effects on blood triglyceride levels in animals are unclear, with increased levels in some research reports, and decreased levels in others (Bhathena, 2003). Human studies in this area also report mixed results, with decreased blood levels of total cholesterol and low-density lipoprotein ("bad cholesterol") in some studies but no effect in other studies (Harper, 2006). Thus further research is needed before a recommendation can be postulated in this respect.

In conclusion, the present study indicated clear inhibitory effects of dietary crude Egyptian flaxseeds oil on post-initiation stages of rat colon carcinogenesis and colon tumors. The mechanism proposed here is likely to be through the antiproliferative properties of ω-3 polyunsaturated fatty acid found in α-linolenic acid and flax lignans both found in flaxseeds oil. The study also suggests a promising approach on the modulatory effects of flaxseeds oil on the mucin production properties in the colonic mucosa treated with DMH.

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