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Phytic Acid Protects the Formation of Colonic Aberrant Crypt Foci Induced by Azoxymethane in Male F344 Rats

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랫드에서 azoxymethane으로 유도된 대장 전암병변에 대한 피티산의 방어 효과

허진주 · 이예은 · 이기남 · 남상윤 · 안병우 · 윤영원 · 이범준*

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ABSTRACT – Phytic acid (PA) (Inositol hexaphosphate, IP_6) is a naturally occurring polyphosphorylated carbohydrate that is present in substantial amounts in almost all plants and mammalian cells. Recently PA has received much attention for its role in anticancer activity. In the present study, the preventive effects of PA on colon carcinogenesis were investigated. Six-week old Fisher 344 male rats were fed a AIN-93G purified diet and PA (0.5% or 2% PA in water) for 8 weeks. The animals received two (1^{st} and 2^{nd} week) injections of azoxymethane (AOM, 15 mg/kg b.w.) to induce colonic aberrant crypt foci (ACF). After sacrifice, the total numbers of aberrant crypts (AC) and ACF in colonic mucosa were examined after staining with methylene blue. Blood and serum were analyzed with a blood cell differential counter and an automatic serum analyzer. AOM induced the total numbers of 142.3 ± 22.3 ACF/colon and 336.6 ± 55.1 AC/colon. PA at the doses of 0.5 and 2% decreased the numbers of ACF and AC/colon in a dose-dependent manner. The numbers of ACF/colon and AC/colon by PA at the dose of 0.5% were 124.4 ± 28.5 and 302.7 ± 67.3, respectively. PA at the dose of 2% significantly decreased the ACF and AC numbers to 109 ± 18.1 and 254.8 ± 50.6, respectively ($p < 0.01$). Especially, 2% PA significantly reduced the number of large ACF (≥ 4 AC/ACF) from 26.8 ± 6.2 ACF/colon to 15 ± 6.7 ACF/colon ($p < 0.01$). Although some parameters in blood counts and serum chemistry were changed compared with the control, no specific toxicity was found. These findings suggest that phytic acid can be a chemopreventive agent for colon carcinogenesis resulting from inhibition of the development of ACF in the F344 rat.

Key words: Azoxymethane, Aberrant crypt foci, Colon cancer, Iron-overloaded rat, Phytic acid

Introduction

The incidence and prevalence of the colon cancer are variable among different human populations. Colorectal cancer is one of the leading causes of cancer mortality in both men and women in the United States[1]. In Korea, the incidence and mortality of CRC gradually increased in the last decade, being the fourth leading cause of cancer deaths[2]. Epidemiological studies have shown that high fiber foods, such as fruits, vegetables, whole grains and cereals, may protect against colorectal cancer[3-4].

Phytic acid (PA, inositol hexaphosphate) is a naturally occurring substance that is present in most legumes, including corn, soy beans, wheat bran and nuts. PA consists of a myo-inositol ring with six phosphate moieties attached[5]. PA and its lower phosphorylated forms are also found in most mammalian cells, where they assist in regulating a variety of important cellular functions[6]. It was recognized to possess multiple biological functions. PA can exert its biological effect through its antioxidant properties. PA forms a divalent cation chelate, preventing the formation of reactive oxygen species responsible for cell injury and

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carcinogenesis[7]. Anticancer action of PA has been demonstrated both in vivo and in vitro. PA appears to be boosting activity in natural killer (NK) cells which are immune system cells that can kill tumor cells[8]. Zhang has shown that PA can increase blood NK cell activity in dimethylhydrazine (DMH)-induced colon tumors and inhibit tumor growth and metastasis in rats[9]. PA induced G1 phase arrest and a significant decrease of the S phase of human colon cancer cell line1[0]. Saied and Shamsuddin[11] have demonstrated that PA upregulates the expression of p53, as well as another tumor suppressor gene, p21 WAF1/Cip1.

Chemically induced cancer is a multistage process, involving initiation, promotion, and progression. The promotion stage involves the selective clonal expansion of the increased cell division and/or decreased cell death (apoptosis). The progression stage involves the development of irreversible cancer growth from the preneoplastic lesions. Initiation occurs through exposure to a carcinogen in cells[12]. This is enhanced by proliferation of fixed DNA damage so that it becomes replicable as a mutation. Reactive oxygen species (ROS) are believed to mediate the activation of such carcinogens through hydroperoxide-dependent oxidation that can be mediated by peroxyl radicals. The presence of carcinogen-DNA adducts and oxidative DNA adducts generated by chemical carcinogens suggest an interactive role of ROS in initiation. ROS, therefore, can have multiple effects in the initiation stage of carcinogen activation, causing DNA damage, and interfering with the repair of the DNA damage[13].

Aberrant crypt foci (ACF) were first reported in the colons of AOM-treated C57BL/6J or CF1 female mice[14], and they have been accepted as preneoplastic lesions of the colon. The growth, morphological and molecular features of ACF support the contention that ACF are putative preneoplastic lesions. The traditional "adenoma-carcinoma" sequence of colorectal carcinogenesis has been extended to "ACF-adenoma-carcinoma" sequence[15]. Under microscopy, aberrant crypts appeared larger and had a thicker epithelial lining compared to normal crypts, and usually gathered into a focus, consisting of aberrant crypts from one to hundreds[16].

The aim of this study was to investigate the effects of PA on the formation of colonic ACF induced AOM in the F344 rats.

Materials and Methods

Materials

Phytic acid (PA) and azoxymethane (AOM) were purchased from Sigma Chemical Company (St Louis, MO, USA). PA was prepared every other day at 0.5 g or 2 g/100 ml in water.

Animals

Male Fisher 344 rats (5 weeks age) were obtained from Central Lab. Animal Inc (SLC Inc., Shizuoka, Japan), housed in stainless steel wire cages (3 rats/cage). The temperature and relative humidity were maintained at 20 ± 2°C and 50 ± 20%. Light and dark cycles were at 12 h each. Rats were allowed access to AIN-93G purified rodent diet (Dyets, Inc., Easton Avenue, Bethlehem, USA) and water was provided ad libitum. All animal experiments were conducted in compliance with "Guide for care and use of Laboratory animals" of Chungbuk National University. During the experimental period, weekly body weight and feed consumptions were recorded.

Experimental design

Twenty mice were assigned to each treatment group, while six mice to normal control group. There were normal control, AOM positive control and two treatment groups (Fig. 1). Rats were treated subcutaneously with AOM (15 mg/kg b.w. in saline) twice at 1st and 2nd week after starting the experiment. The rats in the normal control group were received an injection of saline. The rats were fed with AIN-93G purified rodent diet. The AIN-93G purified rodent diet

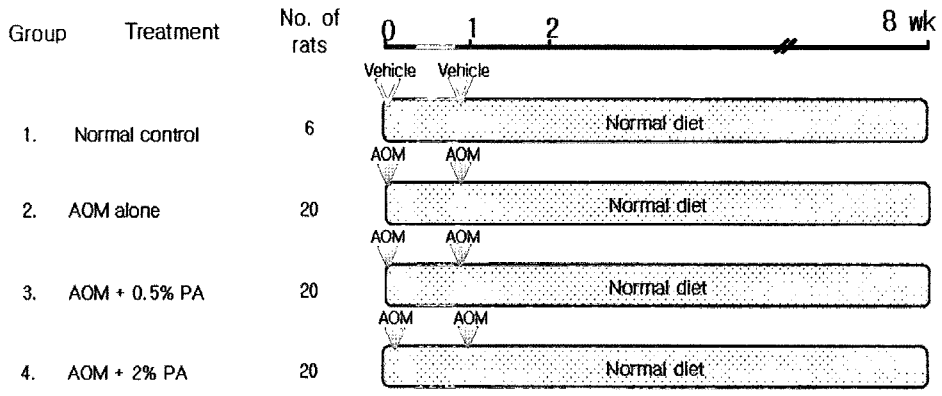

Fig. 1. Experimental design for colon carcinogenesis in F344 rats.

contained 20% casein, 39.7% corstarch, 13.2% dyetrose, 10% sucrose, 5% cellulose, 7% soybean oil, 0.0014% t-butylhydroquinone, 1% vitamin mix, 0.3% L-cystine, 0.25% choline bitartrate, 3.5% salt mix. The rats in two treatment groups received 0.5 or 2.0% PA in drinking water for 8 weeks. The rats in the normal control and AOM alone were received water only.

Sample collection

After laparotomy, blood was collected by a syringe from the abdominal aorta and immediately transferred into tubes containing K_3-EDTA and serum separator tubes (Vacutainer, Becton Drive Franklin Lakes, NJ, USA). The liver, spleen, kidneys and entire large intestine were harvested. One fifth of liver, spleen and kidneys were washed with saline, blotted dry, weighted and then frozen in liquid nitrogen. The remaining tissues were fixed in 10% neutral buffered formalin. The large intestine from cecum to anus was longitudinally opened, flushed with saline, and fixed flat between two pieces of filter paper in 10% neutral buffered formalin.

Blood analyses

Blood samples in EDTA tubes were used for analyses of blood cell counts with Abbott CellDyn-3500 (Abbott Laboratories, Chicago, IL, USA). Blood in serum separator tubes was allowed to clot at room temperature. Serum samples were used for analyses of serum biochemistry with Hitachi-7080 (Hitachi Ltd, Tokyo, Japan).

AC and ACF counts

The colon were removed and flushed with 0.85% NaCl solution and fixed in 10% neutral phosphate buffered formalin. The formalin-fixed colonic tissues were stained in 0.5% methylene blue solution for 30 sec. The total number of ACF and the number of aberrant crypts (AC) in each focus were counted under a microscope (40-100x). ACF were identified with the following morphological characteristics: 1) the enlarged and elevated crypts than normal mucosa and 2) increased pericryptal space and irregular lumens.

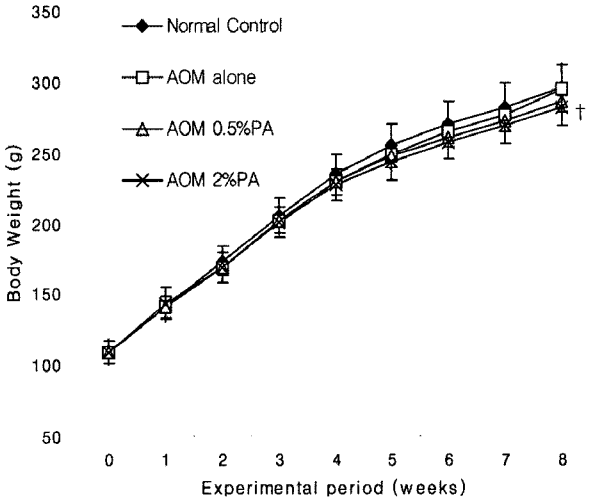

Fig. 3. Changes in body weights of rats. Data represent mean ± S.D. *Significantly different from AOM alone ($p<0.05$).

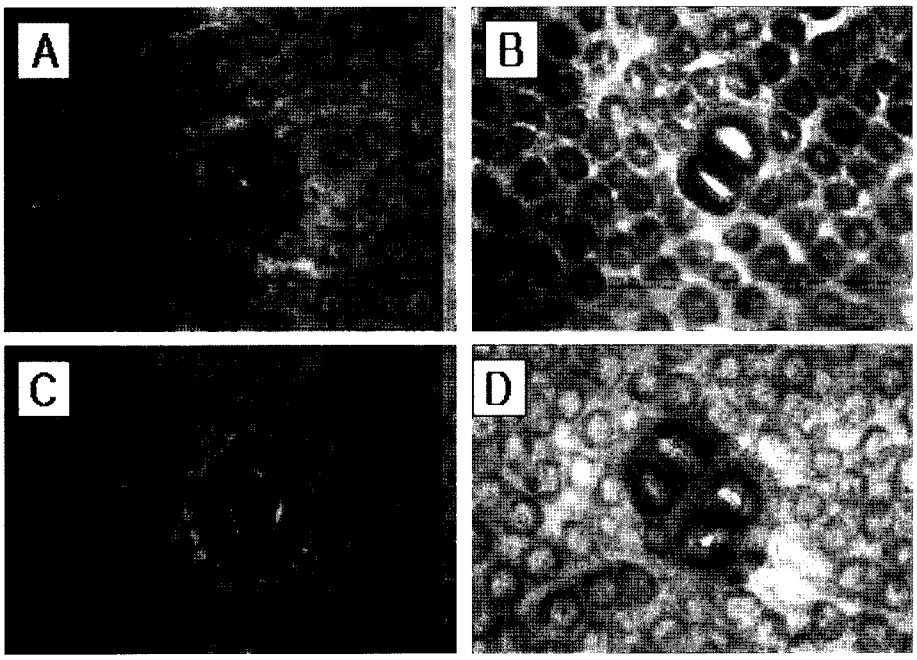

Fig. 2. Representative photographs of aberrant crypt foci with various aberrant crypts. A: one aberrant crypt, B: two aberrant crypts, C: three aberrant crypts, D: Four aberrant crypts.

Statistical analyses

Data were expressed as means ± standard deviation (SD). Data were analyzed with One-way ANOVA and Tukey HSD using SPSS v 12.0 software. The significant differences were statistically determined at the level of $p < 0.05$ or $p < 0.01$.

Results

Changes in weights of body and relative organs

Treatment of PA slightly decreased the body weight of rats. There were significant decreases in body weights of rats in 2% PA at 8th week, compared with AOM alone (Fig. 3). Such changes in the body weight might be due to the significant reduction of feed consumption. The relative liver weights in rats treated with AOM alone were significantly low, compared with the normal control ($p < 0.01$). Treatment of 0.5% PA significantly lowered the relative spleen weight, compared with AOM alone ($p < 0.05$). The relative kidney weight of rats treated with 2% PA was significantly high, compared with AOM alone ($p < 0.01$) (Table 1).

Changes in blood chemistry

There were no significant changes between normal control and AOM alone groups in all blood cell counts. However the treatments of PA significantly increased white blood cell counts, hemoglobin, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, compared with AOM alone (Table 2). The treatment of AOM alone did not change the values of glucose (GLU), glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), total cholesterol (T-CHO), triglyceride (TG), blood urea nitrogen (BUN) in the serum (Table 3). However, creatinine (CRE) value was significantly decreased in AOM alone group compared with normal control ($p < 0.05$). The B/C ratio in AOM alone group was significantly high, compared with the normal control ($p < 0.01$), maybe due to the decreased value of CRE in AOM alone group (Table 3). All of the values except for GLU. in serum were not changed by 0.5% PA. The values of GLU were significantly increased by 0.5% PA, compared with AOM alone ($p < 0.05$). The treatment of 2% PA significantly enhanced CRE, GLU and TG values, compared with AOM alone (Table 3).

Aberrant crypt foci (ACF)

The animals administered saline (vehicle) showed no evidence of ACF formation in the colon. Treatment with

Table 1. Changes of relative organ weights in rats

	Final body weight	Relative organ weight		
		Liver	Spleen	Kidney
Normal control	283.9 ± 17.4	2.73 ± 0.11	0.20 ± 0.00	0.30 ± 0.02
AOM alone	280.0 ± 15.5	2.50 ± 0.12*	0.22 ± 0.02	0.30 ± 0.01
AOM+0.5%PA	274.3 ± 14.1	2.47 ± 0.09	0.20 ± 0.01 §	0.31 ± 0.01
AOM+2%PA	275.6 ± 27.0	2.48 ± 0.20	0.21 ± 0.02	0.32 ± 0.02 §§

Relative organ weight (%) : absolute organ weight/body weight × 100.
Kidney is mean of right and left organs. Data represent mean ± S.D.
*Significantly different from normal control ($p<0.01$).
§ Significantly different from AOM alone ($p<0.05$).
§§ Significantly different from AOM alone ($p<0.01$).

Table 2. Differential blood cell counts in rats

	Normal control	AOM		
		-	0.5% PA	2% PA
WBC (thousands)	4.94 ± 0.85	4.63 ± 0.87	5.62 ± 1.47 §	6.34 ± 1.08 §§
RBC (millions)	9.30 ± 0.48	9.14 ± 0.28	9.09 ± 0.20	9.12 ± 0.17
Hb (g/dl)	15.2 ± 0.6	15.0 ± 0.58	15.4 ± 0.3 §	15.6 ± 0.2 §§
HCT (%)	48.8 ± 2.3	48.7 ± 1.6	48.1 ± 1.0	48.8 ± 0.8
MCV (fl)	52.6 ± 2.5	53.3 ± 2.2	52.9 ± 0.6	53.6 ± 0.8
MCH (pg)	16.3 ± 1.1	16.4 ± 0.7	16.9 ± 0.2 §	17.2 ± 0.2 §§
MCHC (g/dl)	31.0 ± 1.0	30.8 ± 0.7	32.0 ± 0.5 §§	32.0 ± 0.4 §§

WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; HCT, hematocrit MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.
Data represent mean ± S.D.
§ Significantly different from AOM alone ($p<0.05$).
§§ Significantly different from AOM alone ($p<0.01$).

Table 3. Serum biochemistry in rats

	Normal control	AOM		
		-	0.5% PA	2% PA
CRE (mg/dl)	0.60 ± 0.06	0.49 ± 0.07*	0.52 ± 0.05	0.54 ± 0.06 §
GLU (mg/dl)	119.0 ± 22.2	112.3 ± 14.2	127.6 ± 10.1 §	129.6 ± 22.7 §
GOT (IU/L)	122.2 ± 10.5	124.4 ± 14.5	129.1 ± 15.9	122.4 ± 28.2
GPT (mg/l)	57.8 ± 5.0	63.7 ± 8.0	65.6 ± 10.1	70.8 ± 10.2
T-CHO (mg/dl)	65.8 ± 6.3	56.7 ± 4.9	56.4 ± 6.9	58.2 ± 10.8
TG (mg/dl)	84.7 ± 29.7	49.1 ± 15.8	58.9 ± 15.6	72.7 ± 28.4 § §
BUN (mg/dl)	19.2 ± 1.1	20.7 ± 2.5	19.9 ± 2.5	20.5 ± 1.8
B/C ratio	32.2 ± 3.8	43.5 ± 7.9**	38.6 ± 6.6	38.3 ± 4.6

CRE, creatinine; GLU, glucose; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvate transaminase; T-CHO, total cholesterol; TG, triglyceride; BUN, blood urea nitrogen; B/C ratio, BUN/creatinine ratio.
Data represent mean ± S.D
*Significantly different from normal control ($p<0.05$).
**Significantly different from normal control ($p<0.01$).
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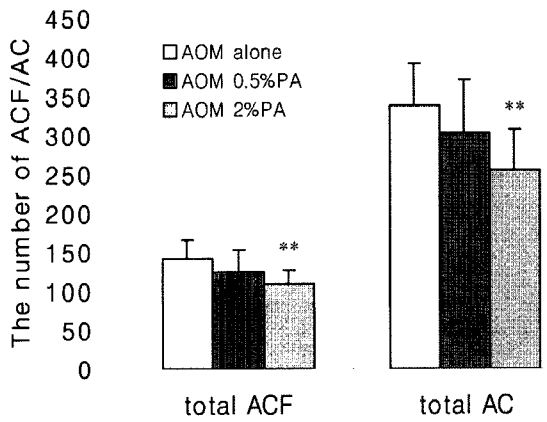

Fig. 4. Effect of phytic acid on formation of aberrant crypts (AC) and foci (ACF) in the colon of F344 rats. Bars represent mean ± S.D.
**Significantly different from AOM alone ($p<0.01$).

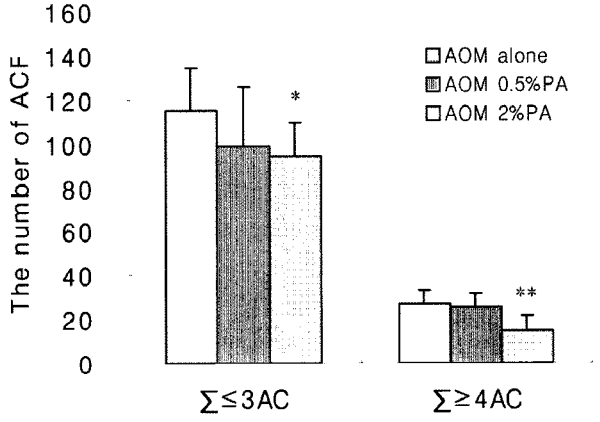

Fig. 5. Effect of phytic acid on formation of aberrant crypts foci (ACF) with various aberrant crypts (AC) in the colon of F344 rats. Bars represent mean ± S.D.
*Significantly different from AOM alone ($p<0.05$).
**Significantly different from AOM alone ($p<0.01$).

AOM induced the total number of 142.3 ± 22.3 ACF/colon which were composed of total number of 336.6 ± 55.1 AC/colon. PA treatments at levels of 0.5% and 2% dose-dependently decreased the total number of ACF/colon to 124.4 ± 28.5 and 109.0 ± 18.1, respectively, and decreased the total number of AC/colon to 302.7 ± 67.3 and 254.8 ± 5 0.6, respectively (Fig. 4). However, only 2% PA had a significant difference, compared with AOM alone ($p < 0.01$). The percentage of reductions of ACF/colon and AC/colon in the treatment of 2% PA were 23% and 24%, respectively ($p < 0.01$). The number of large ACF (≥ 4 AC/ACF), which is suggested to possess a greater tumorogenic potential (McLellan *et al.*, 1991), was strongly suppressed from 26.8±6.2 ACF/colon in AOM alone to 15.0 ± 6.7 in 2% PA-treated group ($p < 0.01$) (Fig. 5). Compared to the AOM alone group, 2% PA treatment decreased the number of large ACF by 44% ($p < 0.01$) and the small ACF (< 3 AC/ACF) by 19% ($p < 0.05$).

Discussion

The objective of the present study was to evaluate protective effect of phytic acid on the formation of colonic aberrant crypt foci (ACF) induced by AOM in male F344 rats. In this study, PA at 2% reduced the body weight gains of rat compared to control group. The reduction of body weight by PA was similar to those in previous studies[17-18].

In this investigation, rats were chosen as experimental animals because ACF have been studied in them. The adoption of short-term colon carcinogenesis model, which uses ACF identification as an endpoint structure, addresses

the need for investigating a phenomenon related to primary prevention of cancer, at a stage when this disturb is not yet phenotypically characterized. Methylene blue-stained whole colon were evaluated for the presence of ACF. PA at the 2% level also significantly decreased the number of ACF induced by AOM in this study. This data was consistent with the results reported by other researchers[17-18]. In addition, the number of large ACF ($\geq$ 4 AC/ACF), which had been suggested to possess a greater tumorogenic potential than small ACF ($\leq$ 3 AC/ACF), was significantly reduced by 2% PA treatment.

In terms of human health, dietary PA might have both negative and positive roles. The effectiveness of PA as a cancer preventive agent was shown in colon cancer induced in different species (rats and mice) with different carcinogens (DMH and AOM)[18-21]. The proposed mechanisms of action are an increase in natural killer cell activity[9,22], alteration in signal transduction[23-24], and antioxidant activity[25]. However, the exact mechanism by which it exerts these effects has yet to be elucidated. The positive effects are interest in the developed world where there is greater concern over pathologies of a aging such as oxidative damage and cancer, whereas the negative effects of dietary PA have their greatest impact on youth and growth in the developing world. PA is a strong chelator of mineral cations such as calcium, iron and zinc, forming mixed salts that are largely excreted by human and other non-ruminant animals. Excretion of dietary PA can contribute to a major public health problem in the developing world - iron and zinc deficiency[26].

However, the chelating effect of PA may contribute to its anticancer activity. An iron-overloaded diet increased the total numbers of preneoplastic lesions (ACF) in the colon carcinogenesis model of rats[27-29]. In addition, phytic acid, a significant component of dietary fiber was found to reverse the augmenting effect of oral iron on tumor yield and incidence[27]. The iron's effect on colorectal tumor induction takes place during the promotional phase of carcinogenesis and not during initiation. These experiments support the epidemiologic observation that dietary iron may augment colorectal cancer risk and that the mechanism by which dietary fiber diminishes colorectal cancer risk may be the chelation of dietary iron by the phytic acid component of dietary fiber2[7].

Wurzelmann *et al.*[30] have reported epidemiological data suggesting that humoral exposure to iron increases the risk of distal carcinoma in humans. Although it is hypothesized that iron has a potentially deleterious effect through its prooxidant capacity, previous studies have so far produced inconsistent results. Intraluminal iron may stimulate an increase in cell proliferation directly, via participation in Fenton reaction and hydrogen peroxide production[31] through an increase in oxidative stress in the dividing cells as a result of hydrogen peroxide exposure to increased cell loss from the luminal surface.

The data of this study suggests that PA can be a chemopreventive agent by many proposed mechanisms in colorectal carcinogenesis. Further studies are required to elucidate the influence of different iron status and/or phytic acid on the colorectal carcinogenesis.

요 약

대장암은 국민경제 수준의 향상과 식생활의 서구화에 따라 급격하게 증가하여, 등록환자 기준으로 2005 년 현재 위암에 이어 두 번째로 많은 상태이다. 특히 육류에 많이 함유된 철분 및 지방의 과다섭취가 주요원인으로 여겨지고 있다. Phytic acid (PA) (Inositol hexaphosphate, IP6) 는 식물의 박류, 콩류 등에 약 0.1-5% 농도로 존재하며, 포유류의 세포에서도 존재한다. PA 는 금속이온과 결합하는 성질을 갖고 있어서, hydroxyl radical 과 같은 활성산소종의 형성을 억제하여 항산화 작용을 나타낸다. 본 연구에서는 PA 가 대장암 발생의 전암병변인 aberrant crypt foci (ACF) 의 발생을 억제하는지를 조사하고자 수컷 F344 랫드를 사용하였다. Azoxymethane (AOM) 을 실험시작 1 주 및 2 번째 주에 투여함으로 ACF 를 유발하였고, 실험기간은 8 주로서 AIN-93G 사료급여와 동시에 음수로서 0.5% 와 2% PA 를 급여하였다. 부검 후, 혈액검사와 대장암 발생의 초기단계인 ACF 를 측정하였다. 결과로서 AOM 투여 대조군과 비교해 볼 때 PA 의 농도가 증가함에 따라 ACF 와 aberrant crypt (AC) 의 수가 감소하였고, 2% PA 는 유의적으로 ACF 와 AC 의 수를 감소시켰으며, 더욱이 4 개 이상 AC 를 갖는 ACF 의 수를 유의적으로 감소시켰다. 또한 혈액 · 생화학적 수치에서 어느 정도 유의적 변화가 나타났지만 정상범위내에서의 변화로서 인정될 수 있을 것이다. 본 실험에서 이러한 결과는 phytic acid 가 대장암 발생과정에 전암병변의 형성을 억제함으로서 최종 대장암발생에 억제효과를 나타낼 수 있을 것이라 사료된다.

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