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Phytic acid does not affect the formation of colonic aberrant crypt foci in Fe-overloaded male F344 rats

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Abstract : There are accumulating evidences that high levels of dietary iron may play a role in colon carcinogenesis. Elevated iron status has been associated with oxidative stress. Phytic acid (PA) functions as an antioxidant by chelating divalent cations and prevents formation of reactive oxygen species responsible for cell injury and carcinogenesis. The protective effect of PA was investigated on formation of aberrant crypt foci (ACF) induced by azoxymethane (AOM) in iron-overloaded male F344 rats. After acclimation with AIN-93G purified diet (35 ppm Fe, normal control diet) for one week, animals were fed iron-overloaded diet (350 ppm Fe) and PA (0.5% or 2% PA in water) for 8 weeks. Animals received two (1st and 2nd week) injections of AOM (15 mg/kg b.w.) to induce colonic ACF. The colonic mucosa was examined for the total numbers of aberrant crypt (AC) and ACF after staining with methylene blue. The blood and serum were analyzed with a blood cell differential counter and an automatic serum analyzer. Iron-overloaded diet increased the concentration of iron in liver of the rats. But iron-related parameters in blood were not changed among experimental groups. The numbers of ACF per colon and AC per colon were 178.8 ± 33.2 and 448.4 ± 110.2 in the iron-overloaded F344 rats. The total AC was significantly increased, compared with normal-diet AOM control group ($p < 0.05$). The treatments of PA at the dose of 0.5% slightly decreased the number of ACF and AC per colon to 153.6 ± 29.5 and 396.3 ± 107.5. However, there were no significant differences in the total numbers of ACF and AC between the AOM control group and PA (0.5% or 2%)-treated groups. These results suggest that PA may not affect the formation of ACF or AC induced by AOM in iron-overloaded F344 rats.

Keywords : aberrant crypt foci, azoxymethane, colon cancer, iron-overloaded rat, phytic acid

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer mortality in the Westernized countries [19]. The incidence and mortality of CRC in Korea gradually increased in the last decade, being the fourth leading cause of cancer deaths [23]. Epidemiological links between consumption of red meats and colon cancer have prompted studies to define the mechanism by which components of red meats, including iron, promote colon tumorigenesis [14, 33, 35, 40]. Red meat consumption is considered to increase gut lumen concentrations of iron, which can lead to reactive oxygen species (ROS) via the Fenton reaction [16]. In biological system, iron bound to low molecular weight chelators such as citrate or ATP can generate ROS via Fenton/Haber-Weiss or autoxidation reactions [30]. ROS may interact with and modify cellular protein, lipid, and DNA, which results in altered target cell function. The consequences of this are probably damage in epithelial cells of the colon mucosa [2]. Oxidative DNA damage may participate in ROS-

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induced carcinogenesis [10].

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 decrease 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 [13]. This is enhanced by proliferation of fixed DNA damage so that it becomes replicable as a mutation. 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 [21].

Aberrant crypt foci (ACF) were first reported in the colons of azoxymethane (AOM)-treated C57BL/6J or CF1 female mice [4], and they have been accepted as preneoplatic 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 [5]. Under microscope, 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 [26].

Phytic acid (PA) (Inositol hexaphosphate, IP6) is a naturally occurring substance that is present in most legumes, including corn, soy beans, wheat bran and nuts [11]. PA consists of a myo-inositol ring with six phosphate moieties attached [11]. PA and its lower phosphorylated forms are also found in most mammalian cells, where they assist in regulating a variety of important cellular functions [39]. It is believed to be the active ingredient that gives these substances their cancer fighting abilities [11]. PA may exert its greatest biologic effect through its antioxidant properties. PA forms an iron chelate which inhibits iron mediated oxidative reactions and limiting site specific DNA damage [27]. 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 cell that can kill tumor cells [36]. Zhang has shown that PA can increase blood NK cell activity in dimethylhydrazine-induced colon tumors in rats and inhibit tumor growth and metastasis in rats [44]. PA induced G1 phase arrest and a significant decrease of the S phase of human colon cancer cell line [9]. Saied and Shamsuddin [34] have demonstrated that PA upregulates the expression of p53, as well as another tumor suppressor gene, p21 WAF1/Cip1.

The large fraction of dietary iron that remains unabsorbed in the small intestine may enter colon and participate, in conjuction with the intraluminal bacteria, in Fenton-type reactions, which increase the production of hydrogen peroxide and hydroxyl radicals at the mucosal surface. Hydrogen peroxide and iron may enter the colonocytes and increase the risk of DNA damage, resulting in promotion of colon tumor. In this study we investigated the effects of dietary iron supplementation with phytic acid on formation of ACF induced by AOM in male F344 rats

Materials and Methods

Materials

PA and AOM were purchased from Sigma Chemical Company (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 old) were obtained from SLC Inc. (Japan). The temperature and relative humidity in animal facility were maintained at 21 ± 1°C and 50 ± 10%. Light and dark cycles were at 12 h each. Rats were allowed access to AIN-93G purified rodent diet (Dyets, USA) and water was provided ad libitum. After acclimatization for one week, the animals were then taken off chow feed, and placed on iron-modified diet. All animal experiments were conducted in compliance with "Guide for care and use of Laboratory animals" of Chungbuk National University (IACUC Approved No. SPF-06-36) During the experimental period, weekly body weight and feed consumptions were recorded.

Experimental designs

Twenty mice were assigned to each treatment group, while six mice to normal control group. Rats were treated twice subcutaneously with AOM (15 mg/kg

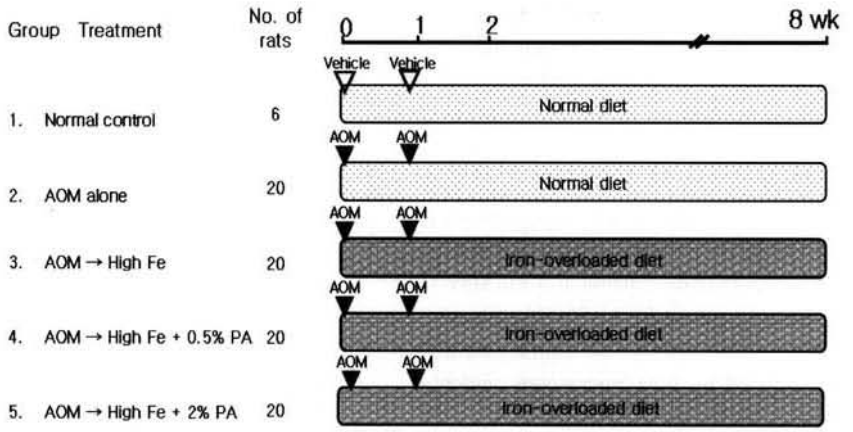

Fig. 1. Experimental design for colon carcinogenesis in iron-overloaded F344 rats.

b.w.) in saline at 6th and 7th weeks of age. The rats in the normal control group received an injection of saline. The experimental period was 8 weeks. There were five experimental groups including normal control, AOM alone positive control, AOM + Fe-overloaded group (HFe) and two treatment groups (AOM + HFe + 0.5%PA and AOM + HFe + 2.0%PA) (Fig. 1). The rats in the normal control and AOM alone groups were fed AIN-93G purified rodent diet (normal diet, 35 ppm iron) and the rats in the other three groups were fed iron-overloaded diet (HFe, 350 ppm iron). The rats in two treatment groups received 0.5 or 2.0% PA in drinking water. The rats in the normal control, AOM alone, and AOM + HFe groups were received water only.

Table 1. Composition of experimental diets

Ingredient (g/kg diet)	Normal diet	Fe-overloaded diet
Casein	200	200
Cornstarch	397	100
Dyetrose	132	132
Sucrose	100	334
Cellulose	50	50
Soybean oil	70	70
t-Butylhydroquinone	0.014	0.014
Vitamin mix	10	10
L-Cystine	3	3
Choline bitartrate	2.5	2.5
Salt mix	35	35
Fe premix		63

Experimental diets

The AIN-93G purified rodent diet 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 iron-overloaded diet was made by adding Fe premix to normal diet. Thus, the normal diet contained 35 ppm iron and the iron overloaded diet contained 350 ppm iron (Table 1).

Sample collection

At 14 weeks of age, all rats were sacrificed. 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, 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 complete blood cell count with Abbott CellDyn-3500 (Abbott Laboratories, 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, Japan).

Perls' Prussian blue staining in liver

For visualization of colonic iron absorption, Perls' Prussian blue staining for ferric ion was performed on formalin-fix, paraffin-embedded liver section. Paraffin-embedded liver sections were deparaffinized, rehydrated, and incubated in a ferrocyanide solution (20% hydrochloric acid and 10% potassium ferrocyanide) (Sigma Chemical Company, USA) at 60 for 20 min. 0.1% Nuclear fast red (Sigma Chemical Company, USA) was used as a counterstain in 5% aluminum sulfate solution. The relative amount of staining from each animal was assessed by light microscopy under × 100 magnification.

Iron analyses in liver

For the determination of total iron, samples of liver were analyzed by ICP-AES (Inductively coupled plasma spectrophotometer) (JY 38 Plus, JOBIN-YVON, France). Samples of the frozen material were digested and ashed at 200°C for 4 h using concentrated nitric acid and hydrogen peroxide. For the ICP-AES analysis of iron, the digested sample was diluted with equal amounts of de-ionized water before analysis.

Aberrant crypts (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 AC in each focus were counted under a microscope (× 40 – 100). 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.

Statistical analysis

Data were expressed as means ± 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

General observations

The iron-overloaded diet slightly decreased the body weight. In addition, there were a significant reduction in body weight at 7 and 8 week, compared with AOM alone ($p < 0.05$) (Fig. 2). The body weight were not changed by PA treatments, compared with iron-overloaded control group. There were no differences in relative liver, spleen, and kidney weights between normal control and iron-overloaded groups (Table 2). Treatment of 2% PA was significantly increased in the relative weight of kidneys ($p < 0.01$).

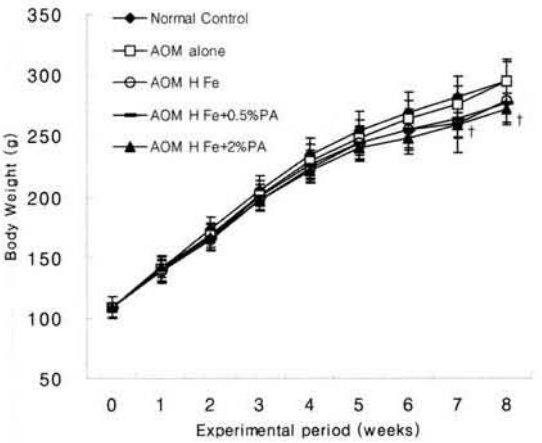

Fig. 2. Changes of the body weight in iron-overloaded F344 rats. †Significantly different from Azoxymethane (AOM) alone ($p < 0.05$).

Blood analyses

There were no significant differences in white blood cell (WBC), red blood cell (RBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration (MCHC) by high iron diet, compared with AOM alone (Table 3). However, WBC count and MCHC value were significantly enhanced in 2% PA group, compared with iron-overloaded control group ($p < 0.05$).

The values of glucose and triglyceride in serum were significantly augmented by iron-overloaded diet, compared with AOM alone ($p < 0.01$) (Table 4). The iron-overloaded diet also significantly decreased the values of blood urea nitrogen/creatinine (B/C) ratio ($p < 0.01$). Meanwhile, treatments of PA had no significant effect in serum analyses, compared with iron-overloaded control group.

Iron concentration in liver

Iron store in the liver was estimated by inductively coupled plasma spectrophotometer (ICP-AES) and

Table 2. Changes of relative organ weights in iron-overloaded 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 ± 14.1	2.50 ± 0.12	0.22 ± 0.02	0.30 ± 0.01
AOM HFe	266.5 ± 18.6	2.59 ± 0.25	0.22 ± 0.01	0.30 ± 0.02
AOM HFe +0.5%PA	264.2 ± 15.8	2.61 ± 0.37	0.22 ± 0.01	0.31 ± 0.02
AOM HFe +2%PA	260.5 ± 13.2	2.52 ± 0.13	0.21 ± 0.01	0.33 ± 0.02†

Relative organ weight (%) : absolute organ weight / body weight × 100.
Kidney is mean of right and left organs.
*AOM: azoxymethane.
†Significantly different from AOM + HFe ($p < 0.05$).

Table 3. Differential blood cell counts in iron-overloaded F344 rats

	Normal control	Azoxymethane (AOM)			
		–	HFe	HFe + 0.5%PA	HFe + 2%PA
WBC (thousands)	4.94 ± 0.85	4.63 ± 0.87	5.57 ± 1.38	5.50 ± 1.23	6.78 ± 1.44*
RBC (millions)	9.30 ± 0.48	9.14 ± 0.28	9.11 ± 0.30	9.11 ± 0.11	9.16 ± 0.26
Hb (g/dl)	15.1 ± 0.6	15.0 ± 0.6	14.9 ± 0.8	14.7 ± 1.5	15.6 ± 0.7
HCT (%)	48.8 ± 2.3	48.7 ± 1.6	46.8 ± 1.6	46.2 ± 3.6	47.6 ± 1.5
MCV (fl)	52.6 ± 2.5	53.3 ± 2.2	51.4 ± 1.8	50.7 ± 2.7	51.8 ± 1.7
MCH (pg)	16.3 ± 1.1	16.4 ± 0.7	16.4 ± 0.8	16.2 ± 1.3	16.9 ± 0.7
MCHC (g/dl)	31.0 ± 1.0	30.8 ± 0.7	31.9 ± 0.7	31.8 ± 1.0	32.7 ± 0.7*

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.
*Significantly different from AOM+LFe ($p < 0.05$).

Table 4. Serum biochemistry of iron-overloaded F344 rats

	Normal control	Azoxymethane (AOM)			
		–	HFe	HFe + 0.5%PA	HFe + 2%PA
CRE (mg/dl)	0.60 ± 0.06	0.49 ± 0.07	0.53 ± 0.06	0.56 ± 0.06	0.51 ± 0.05
GLU (mg/dl)	119.0 ± 22.2	112.3 ± 14.2	134.9 ± 13.7*	125.6 ± 15.3	141.3 ± 14.9
GOT (IU/l)	122.2 ± 10.5	124.4 ± 14.5	115.1 ± 18.0	118.0 ± 20.4	120.9 ± 19.7
GPT (mg/l)	57.8 ± 5.0	63.7 ± 8.0	58.8 ± 8.4	63.1 ± 9.9	64.9 ± 7.4
T-CHO (mg/dl)	65.8 ± 6.3	56.7 ± 4.9	58.4 ± 8.7	59.4 ± 17.5	59.8 ± 8.0
TG (mg/dl)	84.7 ± 29.7	49.3 ± 15.8	72.3 ± 17.7*	67.6 ± 24.3	87.3 ± 16.3
BUN (mg/dl)	19.2 ± 1.1	20.7 ± 2.5	19.0 ± 1.3	18.6 ± 2.4	18.5 ± 1.7
B/C ratio	32.2 ± 3.8	43.5 ± 7.9	36.1 ± 4.1*	33.8 ± 5.3	37.0 ± 5.2

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.
*Significantly different from AOM alone ($p < 0.01$).

Prussian blue staining. Iron-overloaded diet increased liver iron concentration, regardless of PA treatments (Fig. 3). In Prussian blue iron staining, numerous iron deposits were detected in the liver of rats fed the iron-overloaded diet (Fig. 4).

Formation of aberrant crypt foci

Iron-overloaded diet significantly increased the

number of AC/colon from 336.58 ± 55.1 to 448.42 ± 110.2, compared with AOM alone ($p < 0.05$) (Fig. 5). The number of AC/colon in iron-overloaded was 33% higher than that found in the AOM alone. The number of ACF/colon was slightly increased, however there were no significant differences, compared with AOM alone group. PA treatment at levels of 0.5% and 2% had no significant changes on the formation of ACF/colon and AC/colon, compared with iron-overloaded control group. Although high iron diet slightly enhanced the number of large ACF (≥ 4 AC/ACF) and small ACF (≤ 3 AC/ACF), there were no significant differences, compared with AOM alone (Fig. 6). PA treatments had no changes on the number of ACF in iron-overloaded rats.

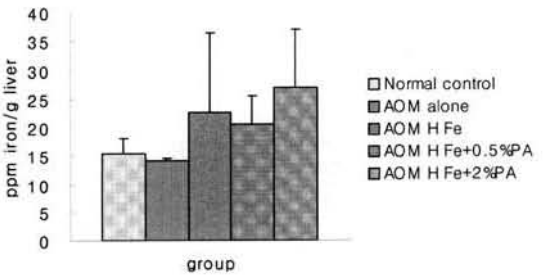

Fig. 3. Hapatic iron levels in F344 rats. Each bar represents the mean value ± SD.

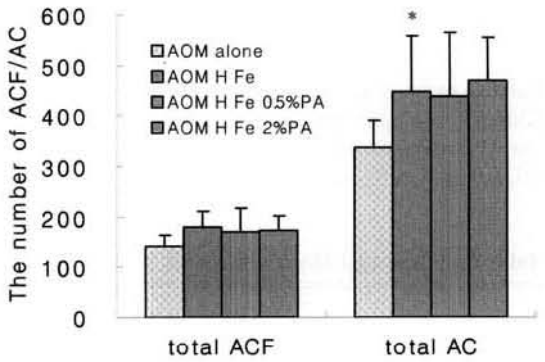

Fig. 5. Effect of phytic acid on formation of aberrant crypts and foci in the colon of iron-overloaded F344 rats. Each bar represents the mean value ± SD. *Significantly different from AOM alone ($p < 0.05$).

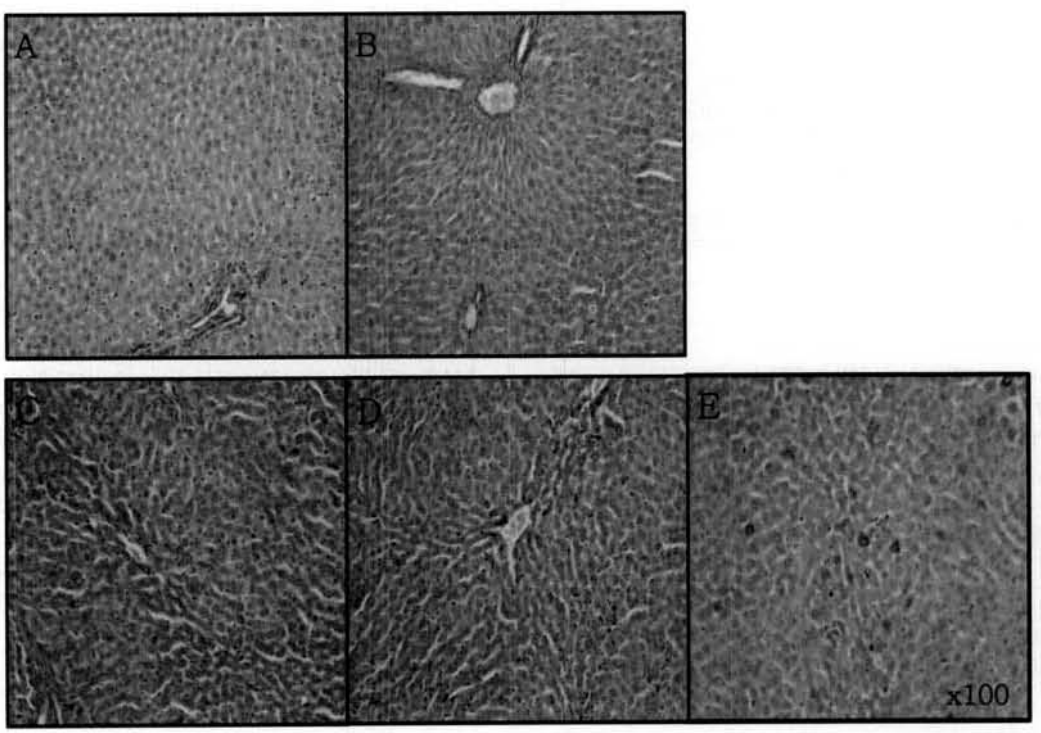

Fig. 4. Perls' Prussian blue staining for hepatic iron in F344 rats. Hepatic iron deposits were rare in normal control and Azoxymethane (AOM) alone groups in which rats fed the normal iron diet (A, B). Numerous iron deposits were detected in HFe groups in which rats fed iron-overloaded diet (C, D and E). Normal control (A), AOM alone (B), AOM + HFe (C), AOM + HFe + 0.5%PA (D), AOM + HFe + 2%PA (E).

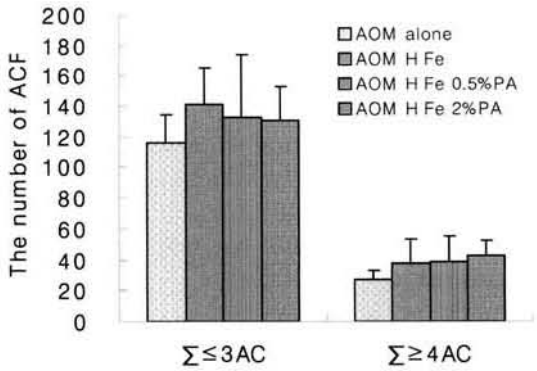

Fig. 6. Effect of phytic acid on formation of colonic aberrant crypts (≥4 ACs) in Fe-overloaded F344 rats. Each bar represents the mean value ± SD.

Discussion

The present study was designed to evaluate the relation between dietary high iron intake and colon carcinogenesis, and to investigate the protective effect of phytic acid on the formation of colonic ACF induced by AOM in male F344 rats. High iron diets contained 350 ppm Fe that was approximately 10-fold higher than the levels found in normal iron diet (35 ppm). Dietary iron supplementation elevated the liver iron concentration, but not blood parameters such as RBC, hemoglobin, hematocrit, and MCV, etc. Although high dietary iron intake significantly increased formation of AC in the colon of rats compared with normal diet group, PA treatments had no protective effect on the ACF and AC in the present study.

Methylene blue-stained whole colon were evaluated for the presence of ACF. The total numbers of AC in the groups receiving AOM were increased by the iron-overloaded diet. Similarly, Liu *et al.* [24] reported that high iron diet with 500 mg Fe/kg elevated the number of ACF in dimethylhydrazine-treated rats. Davis and Feng [6] showed that a 140 μg Fe/g diet enhanced the number of ACF in rats. Wurzelmann *et al.* [43] have reported epidemiological data suggesting that humoral exposure to iron increases the risk of distal carcinoma in humans. However, the relation between high iron and the risk of colorectal cancer is still a controversial issue. 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 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 [8]. In addition, Babbs [1] proposed that generation of hydroxyl radicals via Fenton reaction may increase the conversion of pro-carcinogens to carcinogens in luminal contents. In *in vitro* study, high concentration of iron resulted in formation of ROS, and the carcinogenic risks from ferric iron enhanced tumor cell growth and caused progenotoxic effects [22]. On the other hand, Soyars *et al.* [41] reported that dietary iron did not enhance oxidative stress, cell proliferation and ACF development in the colon of SD rats. Ilsley *et al.* [18] suggested that the effect of iron is limited to the promotional stage of colon tumorigenesis. From his results, dietary iron did not enhanced the number of ACF, whereas high iron increased the size and multiplicity of adenoma compared with low-iron group. Meanwhile, low-iron intake did not prevent the tumor development, but reduced tumor size [15].

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 (1,2-dimethylhydrazine and azoxymethane) [20, 31, 38, 42]. The proposed mechanisms of action were an increase in natural killer cell activity [3, 44], alteration in signal transduction [7, 17], and antioxidant activity [12]. 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 [32].

In the present study, PA has no effects on the formation of ACF in iron-overloaded status. It is not clear whether PA was not enough to chelate iron to restrict its availability in the intestine of rats. Previous study have shown that a fourfold increase in dietary

iron causes significant increases in iron in the large bowel of the rat. This iron was associated with changes in crypt cell proliferation, but there was no evidence of an effect of PA on crypt cell proliferation [25].

The present study suggests that dietary high iron intake are risk factors for promoting colorectal cancer, but PA in the high iron status can not protect the ACF formation in F344 Rats. Further studies are required to elucidate the mechanisms of iron status and phytic acid on the colorectal carcinogenesis.

Acknowledgments

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