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Protective Effect of Selenium on Experimental Colon Carcinogenesis in Mice Fed a Low Iron Diet

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ABSTRACT - Selenium (Se) is known to prevent from several cancers, while iron (Fe) is known to be associated with high risk of cancers. The role of Se on colon carcinogenesis was investigated in an animal model induced by azoxymethane (AOM) and dextran sodium sulfate (DSS) in low Fe mice. Six-week old ICR mice fed on a low Fe diet (4.5 ppm Fe; generally 10 times lower than normal Fe) with three different Se (0.02, 0.1 or 0.5 ppm) levels for 24 weeks. The animals received weekly three (0~2nd weeks) i.p. injections of AOM (10 mg/kg B.W), followed by 2% DSS with drinking water for 1 week to induce the colon cancer. There were five experimental groups including vehicle, positive control (normal Fe level, AOM/DSS), Low Fe (LFe) + AOM/DSS+Low Se (LSe), LFe + AOM/DSS + medium Se (MSe) and LFe + AOM/DSS + high Se (HSe) groups. HSe group showed a 66.7% colonic tumor incidence, MSe group showed a 69.2% tumor incidence, and LSe group showed a 80.0% tumor incidence. The tumor incidence was negatively associated with Se levels of diets. Tumor multiplicity in Hse group was significantly low compared to the other groups ($p < 0.05$). With increasing Se levels of diets, the primary anti-proliferating cell nuclear antigen (PCNA)-positive cells were decreased and apoptotic bodies were increased in a dose-dependent manner. Se-dependent glutathione peroxidase activity and its protein level were dependent on the levels of Se of diets. Malondialdehyde level in liver was lowest in Hse group among experimental groups. These findings indicate that dietary Se is chemopreventive for colon cancer by increasing antioxidant activity and decreasing cell proliferation in Fe-deficient mice.

Key words: Selenium, Iron, Colon cancer, Azoxymethane, Dextran sodium sulfate

In the developed countries, colorectal cancer (CRC) is one of the commonest non-smoking related cancers[1]. In the United States, CRC is the third leading cause of cancer-related death in men and women[2]. For inflammatory bowel disease (IBD) and IBD-related CRC, several animal models have been reported and the azoxymethane (AOM) and dextran sodium sulfate (DSS)-induced mouse model is one of the most widely used[3]. A novel colitis-related mouse CRC model initiated with AOM and promoted with DSS was developed to obtain a better understanding of the pathogenesis of CRC[1,4].

Environmental factors have been identified to play the most important roles in the development of this disease, in particular diet and its specific components. These dietary associations with colon cancer have been explained by many epidemiological studies and the carcinogenesis studies have also found a large number of diverse chemicals which can markedly inhibit the development of colon cancer in rodents[5]. Selenium (Se) is an important micronutrient engaged in the protection of colonic cells against a wide range of external and internal stressors[6]. Several animal studies showed the protective effects of Se against aberrant crypt formation and colon tumor development[7-8]. Se occurs naturally in organic forms such as selenomethionine, Se-methylselenomethionine, selenocysteine, and selenocystine and inorganic forms as selenite and selenate. Selenite is greater than 80-percent bioavailable and selenomethionine or selenate can be greater than 90-percent bioavailable[7,10]. Se is essential for selenoprotein synthesis and function. Selenoproteins are also known to play roles in carcinogen metabolism, in the control of cell division, oxygen metabolism, detoxification processes, apoptosis induction and the functioning of the immune system[11]. Se-dependent glutathione peroxidase (GPx) is one of the important enzymes that reduce hydrogen peroxide and a variety of organic hydroperoxides[10].

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Selenomethionine as Se sources is being incorporated non-specifically into the protein pools before being converted into the precursor available for GPx synthesis whereas inorganic Se sources directly enter into the Se pool available for synthesis of selenoproteins[12-13].

Proposed anti-carcinogenic pathways of Se include the repair and prevention of oxidative damage, alteration of metabolism of carcinogenic agents, regulation of immune response, and P53-independent apoptosis, and repair of DNA damage. It is likely that Se acts as an anti-carcinogen through several mechanisms, which vary in importance based on disease status of the individual[14]. Free radicals are natural by-products of oxygen metabolism that may contribute to the development of chronic diseases such as cancer and heart disease. The antioxidant properties of Se can help protect the body from damaging effects of free radicals[15].

Iron (Fe) is essential for normal cellular function, but an excessive amount of Fe is now known to be potentially harmful, by increasing oxidative damage to membranes as a result of the Fenton reaction[16]. Oxidative damage to DNA and other macromolecules appears to play a major role in aging and degenerative diseases such as cancers[17]. Epidemiologic studies have shown a modest association between red meat intake, the major source of dietary Fe, and risk of colon and colorectal cancer[18-19].

Many studies have shown the role of Se or Fe in the colon carcinogenesis, respectively. But, it is the first time to investigate the protective role of Se on the colon carcinogenesis induced by AOM and DSS in mice fed a low Fe diet. The aims of this study were to elucidate how the low Fe levels influence colon carcinogenesis induced by AOM followed by DSS in mice, and to investigate the effects of Se on colon carcinogenesis in mice fed the low Fe diet.

materials and Methods

Materials

Azoxymethane (AOM) was purchased from the Sigma Chemical Company (St Louis MO, USA) and dextran sodium sulfate (DSS) (molecular weight 36,000~50,000) was manufactured by MP biomedicals (Solon, OH, USA).

Animals

Male ICR mice (5 weeks old) were obtained from Central Lab. Animal Inc (SLC Inc., Shizuoka, Japan) and housed in polycarbonate cages (5 mice/cage). The temperature and relative humidity were maintained at 20 ± 2°C and 50 ± 20%, respectively. A light and dark cycle was at 12 h each. Mice were allowed access to AIN-93G purified rodent diet (Dyets, Inc., Easton Avenue, Bethlehem, USA) and water was provided *ad libitum*. The animal experiments were conducted in accordance with "Guide for care and use of Laboratory animals" of Chungbuk National University. After one week of acclimatization, the animals were then taken off chow feed and fed on normal or Fe-deficient diet. During the experimental period, body weight and feed consumption were recorded weekly.

Experimental diets

The AIN-93G purified rodent diet contained 20% casein, 9.7% sucrose, 0.3% L-cystine, 39.7% cornstarch, 13.2% dyetrose, 7% soybean oil, 0.0014% t-butylhydroquinone, 5% cellulose, 3.7% mineral mix, 1% vitamin mix, 0.25% choline bitartrate (Table 1). Normal Fe diet contained 0.9% ferric citrate and low Fe diet contained 0.09% ferric citrate (Table 1). Low Se diet (LSe), medium Se (MSe), and high Se (HSe) diets contained 0.002%, 0.01% and 0.05% Na selenate, respectively. Total weight of the diet was to make 1.0 kg with sucrose.

Experimental designs

Twenty mice were assigned to each experimental group, except for ten mice to vehicle group. There were five experimental groups including vehicle control, positive control (AOM/DSS, normal Fe), low Fe (LFe) + AOM/DSS + low Se (LSe) (LFe + AOM/DSS + LSe), LFe + AOM/DSS + medium Se (MSe) (LFe + AOM/DSS + MSe), LFe + AOM/DSS + high Se (HSe) (LFe + AOM/DSS + HSe) groups (Fig. 1). The animals were treated intraperitoneally with AOM (10 mg/kg body weight in saline) weekly for 3 weeks. Additionally the animals received 2% DSS in the drinking water for 7 days at

Table 1. Composition of experimental diets

Groups	LFe + LSe	LFe + MSe	LFe + HSe
Ingredients	g/kg	g/kg	g/kg
Casein	200	200	200
L-Cystine	3	3	3
Sucrose	97.08	97.0	96.6
Cornstarch	397.486	397.486	397.486
Dyetrose	132	132	132
Soybean Oil	70	70	70
t-Butylhydroquinone	0.014	0.014	0.014
Microcrystalline Cellulose	50	50	50
Mineral Mix #215020 (Rx grade, no Fe Se)	37	37	37
Ferric Citrate P.M 5 mg/g Fe	0.9	0.9	0.9
Na Selenate P.M 1 mg/g Se	0.02	0.1	0.5
Vitamin Mix #310025	10	10	10
Choline Bitartrate	2.5	2.5	2.5
Total	1000	1000	1000

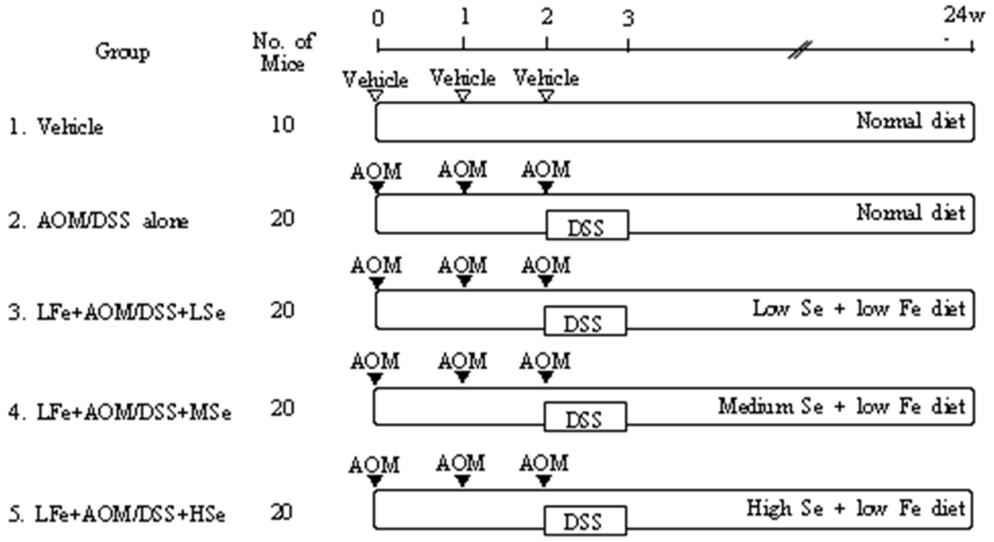

Fig. 1. Experimental design for colon carcinogenesis in mice fed the low Fe. Animals were treated intraperitoneally with azoxymethane (AOM, 10 mg/kg body weight in saline) weekly for 3 weeks. Therafter the treated mice received 2% DSS in the drinking water for 7 days.

3rd week (Fig. 1). The mice in the vehicle group were injected with saline. The mice in the vehicle control and positive control groups were fed on the AIN-93G purified rodent diet (normal Fe diet, 45 ppm Fe) and the mice in the other groups were fed on a low Fe diet (4.5 ppm Fe) with three levels of Se; a low Se (0.02 ppm), a medium Se (0.1 ppm), and a high Se (0.5 ppm).

Sample collection and blood analysis

At 24 weeks, all mice were sacrificed. Before sacrifice, final body weights were measured. 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, lung, stomach, small intestine 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. A half of the large intestine from cecum to anus was longitudinally opened, flushed with saline, and fixed in 10% neutral buffered formalin. The other half were washed with saline, blotted dry and then frozen in liquid nitrogen. The remaining tissues were fixed in 10% neutral buffered formalin. Blood samples in EDTA tubes were used for analysis of complete blood cell count with Abbott CellDyn-3500 (Abbott Laboratories, Chicago, IL, USA).

Fe and Se analysis in liver

For the determination of total Fe, samples of liver were analyzed by inductively coupled plasma spectrophotometer (ICP-AES) (JY 38 Plus, JOBIN-YVON, rue de Lille, France). Samples of the frozen material were digested and ashed at 200°C for 4 h using concentrated nitric acid and hydrogen peroxide. The digested sample was diluted with equal amounts of de-ionized water before analysis. Also, for the determination of total Se, samples of liver were analyzed by inductively coupled plasma mass spectroscopy (ICP-MS) (820-MS, Varian, Santa Clara, CA, USA). Samples of the frozen material were digested and ashed at 200°C for 4 h using concentrated nitric acid and hydrogen peroxide. The digested sample was diluted with equal amounts of 2% nitric acid before analysis.

Glutathione peroxidase (GPx) activity assay

The GPx activity was measured using a commercial kit (Cayman, MI, USA). This method is based on the principle that GPx can catalyze the action of hydrogen peroxide and glutathione into water and oxidized glutathione. Oxidized glutathione is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The results of this enzymatic assay are given in units of GPx activity per milligram of protein (nmol/mg protein). The extinction coefficient for NADPH at 340 nm is 0.00622 μM^{-1} cm^{-1}. The protein concentrations were determined according to the Bradford method (PRO-MEASURE; iNtRON Biotechnology, Seongnam, Korea).

Western blot analysis

A total of 100 mg large intestine tissues were homogenated with 500 μl of proprep (iNtRON Biotechnology, Seongnam, Korea) and centrifuged at 15,000 × g for 15 min at 4°C. The protein concentration was measured by the Bradford method (PRO-MEASURE; iNtRON Biotechnology, Seongnam, Korea).

A total of 40 μg protein per lane were separated on 12% acrylamide gels and electroblotted onto polyvinylidene fluoride (PVDF) membranes (Hybond-ECL, GE Healthcare, Buckinghamshire, UK). Blots were blocked for 1hr at room temperature with 5% (w/v) bovine serum albumin in tris buffered saline (10 mM Tris (pH 8.0) and 150 mM NaCl) solution containing 0.05% Tween-20. GPx-1 expression levels were assessed with 1:100 diluted rabbit GPx-1 antibody (Abcam, Cambridge, UK). β-Actin expression was evaluated to confirm equal amount of protein loadings by mouse monoclonal β-actin (Santacruz, CA, USA). The membrane was incubated for 2~3hr at room temperature or 4°C overnight with specific antibodys. The blot was then incubated with the corresponding conjugated anti-rabbit or anti-mouse immunoglobin-G horseradish peroxidase (1:4000 dilution, Santacruz, CA, USA). Immunoreactive proteins were detected with the ECL western blotting detection system.

Deternination of malondialdehyde (MDA) in liver

The amounts of MDA contained in the tissue homogenate were measured using commercial ELISA kits (Cayman Chemical Company, Michigan, USA). In brief, a mixture of 100 μl liver homogenate, 500 μl of 30 mg/ml sodium dodecyl sulfate, 2 ml HCl, 300 μl of 10 mg/ml phosphotungstic acid, and 1 ml of 7 mg/ml 2-thiobarbituric acid was incubated in boiling water for 30 min with 95°C. After cooling, 5 ml of butanol was added. The organic layer was collected after centrifuging at 1000 × g for 10 min at 4°C. The absorbance was measured at 532 nm and compare with a standard curve constructed with known concentration of 1,1,3,3,-tetramethoxypropane. The data were represented as nM MDA/g protein.

Tumor incidence and multiplicity

Colonic neoplasms were examined macroscopically by two researchers independently. Tumor incidence was defined as number of mice with tumors per total mice in each group. Tumor multiplicity (mean number of tumors per mouse) was defined as the total number of colorectal tumors divided by total number of mice in each group.

Immunohistochemistry of PCNA and TUNEL

The 4-μm formalin-fixed, paraffin-embedded distal colon sections were subjected to deparaffinization and hydration prior to quenching of endogenous peroxidase activity (3% H_2O_2 in methanol for 15 min). The sections were incubated for 60 min with the primary anti-proliferating cell nuclear antigen (PCNA) mouse monoclonal antibody (Santa Cruz, CA, USA) was applied for 60 min in a 1:200 dilution. Slides were processed with the ABC reagent from Vectastain Elite (Vector Laboratories, Burlingame, CA, USA) using DAB as the substrate. Sections were counterstained with mayer hematoxylin.

Levels of apoptosis in distal colon tissue were determined using the TdT-mediated dUTP nick-end labeling (TUNEL) method. The 4-μm formalin-fixed, paraffin-embedded tissue sections from the distal colon were processed according to manufacturer's instructions for the ApopTag peroxidase in situ Apoptosis Detection Kit (TUNEL; Vector Laboratories, Burlingame, CA, USA). The numbers of nuclei with positive reactivity for PCNA- and TUNEL-immunohistochemistry were counted in a total of 3 × 100 cells in 3 different areas of the colonic cancer and expressed as a percentage (mean ± S.E.)[20].

Statistical analysis

Data were expressed as means ± standard error (SE). Data were analysed by one-way analysis of variance and a significant difference among treatment groups were evaluated by Duncan's Multiple Range Test (DMRT) using SPSS v 12.0 software. The results were considered significant at $p < 0.05$.

Results

Changes in body weights

The weights of body were measured during the experimental period. All AOM/DSS-treated groups showed a decrease in body weight throughout the experimental period compared with vehicle control (Fig. 2). In special at the 4th and the 24th weeks, the body weights of all AOM/DSS-treated groups significantly decreased compared with the vehicle control group ($p < 0.05$). Such decreases in the body weight were associated with the reduction of feed consumption. There were no significant differences in the body weight change among the AOM/DSS-treated groups (Fig. 2).

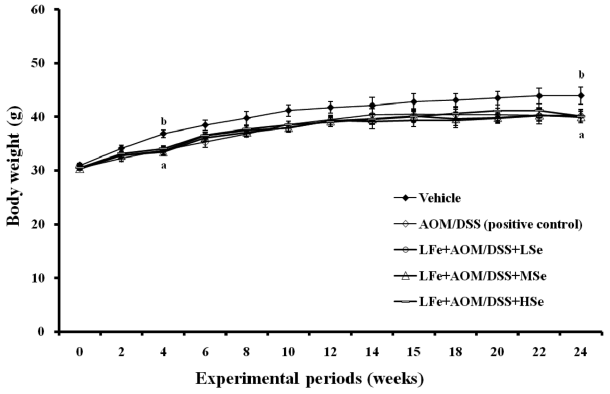

Fig. 2. Change in the body weights of mice. Data represented as mean ± SE. [ab]Means not sharing common superscript letters are significantly different each other at $p<0.05$. AOM: azoxymethane, DSS: dextran sodium sulfate, LFe: low Fe (4.5 ppm) diet, LSe: low Se (0.02 ppm) diet, MSe: medium Se (0.1 ppm) diet, HSe: high Se (0.5 ppm) diet.

Table 2. Differential blood cell counts in ICR mice

	Vehicle	AOM/DSS			
		Positive control	LFe + LSe	LFe + MSe	LFe + HSe
WBC (thousands)	4.61 ± 1.85[b]	2.61 ± 0.61[a]	3.37 ± 1.80[a]	2.17 ± 0.79[a]	2.41 ± 1.35[a]
RBC (millions)	8.72 ± 0.40	7.11 ± 1.66	7.50 ± 3.38	8.26 ± 0.38	7.72 ± 0.85
Hb (g/dl)	13.40 ± 0.60	11.60 ± 1.13	11.10 ± 4.81	12.30 ± 0.67	12.30 ± 0.70
HCT (%)	44.05 ± 2.80	38.20 ± 4.67	36.85 ± 16.05	40.38 ± 2.78	41.53 ± 2.87
MCV (fl)	50.53 ± 0.94[b]	54.50 ± 6.22[b]	49.30 ± 0.85[a]	48.94 ± 3.73[a]	50.95 ± 1.96[b]
MCH (pg)	15.40 ± 0.55[b]	16.65 ± 2.33[b]	14.85 ± 0.35[a]	14.92 ± 0.81[a]	14.90 ± 0.15[a]
MCHC (g/dl)	30.53 ± 1.23	30.81 ± 0.68	30.10 ± 0.14	30.48 ± 0.76	29.63 ± 1.05

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, AOM: azoxymethane, DSS: dextran sodium sulfate, LFe: low Fe (4.5 ppm) diet, LSe: low Se (0.02 ppm) diet, MSe: medium Se (0.1 p pm) diet, HSe: high Se (0.5 ppm) diet. Data represented as mean ± SE. [ab]Means in each column with different superscripts are significantly different ($p < 0.05$).

Change in blood counts

All AOM/DSS-treated groups significantly decreased white blood cells compared with the vehicle control group ($p < 0.05$). There was a significant decrease in the mean corpuscular volume and mean corpuscular hemoglobin in low Fe diet groups as compared to vehicle and positive control groups ($p < 0.05$) (Table 2).

Fe and Se concentration in liver

Low Fe diet groups showed a significant decrease of liver Fe concentrations compared with the vehicle control group ($p < 0.05$) (Fig. 3). However, there was no significant change in the liver Fe concentration among AOM-treated groups.

The Se concentration in liver increased with the diet levels of Se in a dose-dependent manner ($p < 0.05$) (Fig. 4). There was no a significant difference in liver Se concentration among normal or (medium) Se diet groups (Fig. 4).

GPx activity and GPx-1 protein levels in liver

As shown in Fig. 5A, GPx activity in the liver was

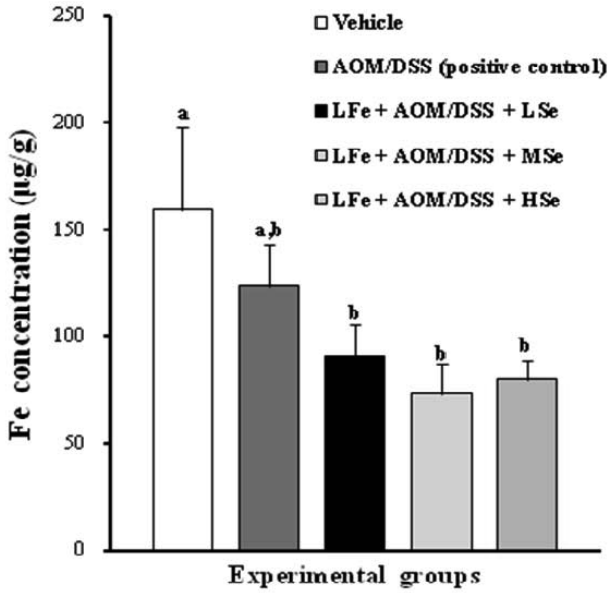

Fig. 3. Hepatic Fe concentration in mice. Fe concentration was determined using an inductively coupled plasma spectrophotometer. Data represented as mean ± SE. [ab]Means not sharing common superscript letters are significantly different each other at $p < 0.05$. AOM: azoxymethane, DSS: dextran sodium sulfate, LFe: low Fe (4.5 ppm) diet, LSe: low Se (0.02 ppm) diet, MSe: medium Se (0.1 ppm) diet, HSe: high Se (0.5 ppm) diet.

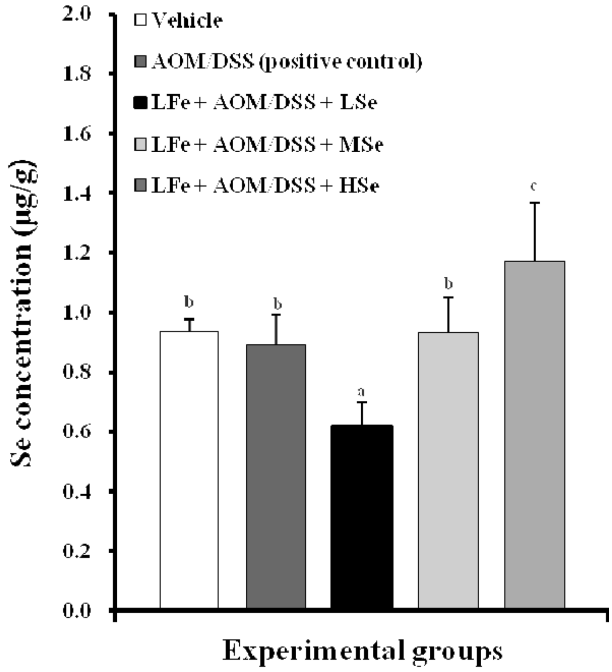

Fig. 4. Hepatic Se concentration in mice. Se concentration was determined using an inductively coupled plasma spectrophotometer. Data represented as mean ± SE. [abc]Means not sharing common superscript letters are significantly different each other at $p < 0.05$. AOM: azoxymethane, DSS: dextran sodium sulfate, LFe: low Fe (4.5 ppm) diet, LSe: low Se (0.02 ppm) diet, MSe: medium Se (0.1 ppm) diet, HSe: high Se (0.5 ppm) diet.

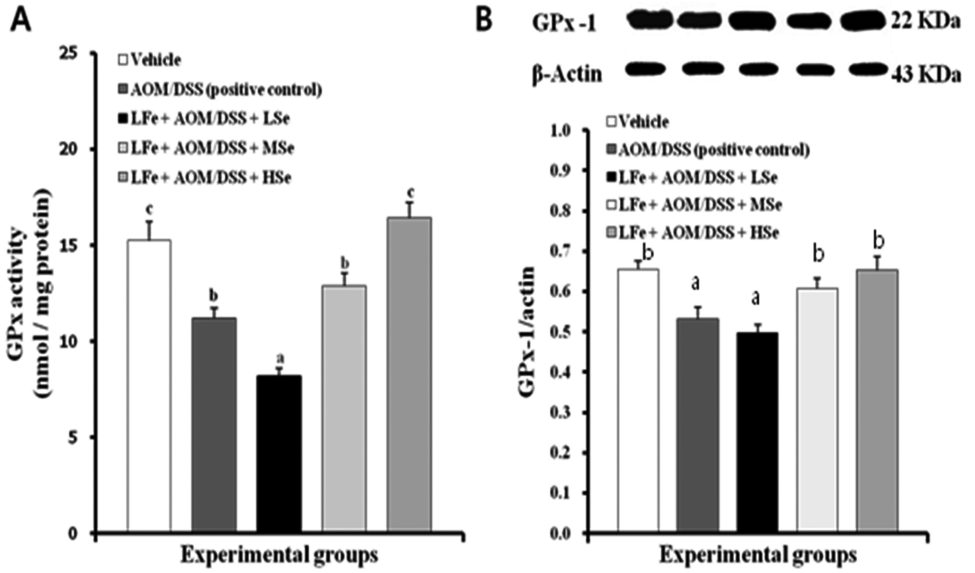

Fig. 5. Activity (A) and protein expression (B) of hepatic glutathione peroxidase (GPx) in mice. Data represented as mean ± SE. [abc]Means not sharing common superscript letters are significantly different each other at $p < 0.05$. AOM: azoxymethane, DSS: dextran sodium sulfate, LFe: low Fe (4.5 ppm) diet, LSe: low Se (0.02 ppm) diet, MSe: medium Se (0.1 ppm) diet, HSe: high Se (0.5 ppm) diet.

significantly dependent on the Se level of diets ($p < 0.05$). The GPx activity in the high Se diet group was significantly high compared with the other AOM/DSS-treated groups ($p < 0.05$).

The expression of GPx-1 was enhanced in the LFe + AOM/DSS + MSe or HSe group compared with the positive control or low Se group ($p < 0.05$) (Fig. 5B). Meanwhile, there was no a significant difference in GPX-1 expression between MSe and HSe groups (Fig. 5B)

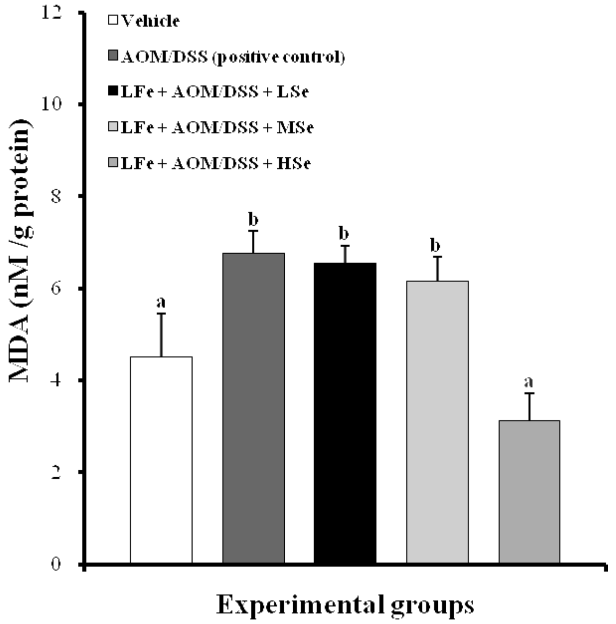

Fig. 6. Hepatic MDA levels in mice. Data represented as mean ± SE. [ab]Means not sharing common superscript letters are significantly different each other at $p < 0.05$. AOM: azoxymethane, DSS: dextran sodium sulfate, LFe: low Fe (4.5 ppm) diet, LSe: low Se (0.02 ppm) diet, MSe: medium Se (0.1 ppm) diet, HSe: high Se (0.5 ppm) diet.

MDA level in liver

As shown in Fig. 6, the level of MDA in liver was significantly decreased in high Se diet group compared with the other groups treated with AOM/DSS ($p < 0.05$).

Tumor incidence and multiplicity

Low Fe diet group (MSe) decreased by 5.8% in tumor incidence compared to positive control (normal Fe diet). The percentage of tumor incidence in mice fed the LSe diet was 10.8% higher and in mice fed HSe diet, it was 2.5% lower than that found in the MSe diet group (Table 3). The HSe diet group showed a significant decrease in tumor multiplicity compared with the other AOM/DSS-treated groups ($p < 0.05$).

Scores of PCNA- and TUNEL-positive cells in colon

The PCNA stain (Fig. 7) and TUNEL stain (Fig. 8) were carried to observe changes in proliferation and apoptosis in colonic mucosa (Table 4). AOM/DSS-treatment significantly increased the proliferation and apoptosis in colonic mucosal cells (Table 4). The level of PCNA protein was significantly down-regulated in the HSe diet group compared with the other AOM/DSS treatment groups (Table 4) ($p < 0.05$). In addition, the HSe diet group exhibited a significantly higher count of brown-color apoptotic bodies compared with the positive control group or the other treatment groups (Table 4) ($p < 0.05$).

Table 3. Effect of Se on colon tumor incidence and multiplicity of mice fed the low Fe diet

Groups	Number of mice		Tumor incidence (%)	Tumor multiplicity (mean ± S.E.)
	Total	Tumor bearing mice		
Vehicle control	10	-	-	-
AOM/DSS alone (positive control)	12	9	75.0	7.63 ± 0.89[a]
LFe + AOM/DSS + LSe	10	8	80.0	6.80 ± 1.20[a]
LFe + AOM/DSS + MSe	13	9	69.2	6.71 ± 0.65[a]
LFe + AOM/DSS + HSe	15	10	66.7	4.57 ± 0.69[b]

LFe: low Fe (4.5 ppm) diet, LSe: low Se (0.02 ppm) diet, MSe: medium Se (0.1 ppm) diet, HSe: high Se (0.5 ppm) diet. The high Se diet group showed a decrease in colon tumor incidence and tumor multiplicity compared with the other AOM/DSS-treated groups. Data represented as mean ± SE. [ab]Means in each column with different superscripts are significantly different ($p < 0.05$).

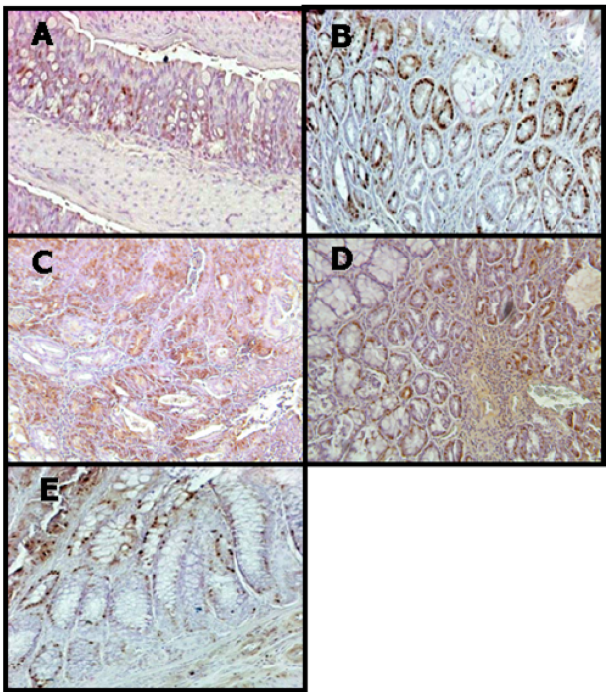

Fig. 7. Immunohistochemistry of PCNA in the colon of mice fed the low Fe diet. The PCNA-positive cells were greatly increased by treatment with AOM/ DSS, which were reduced by co-administration of a high concentration of Se. vehicle control (A), AOM/DSS alone (positive control) (B), LFe + AOM/DSS + LSe (C), LFe + AOM/DSS + MSe (D), LFe + AOM/DSS + HSe (E). (× 100).

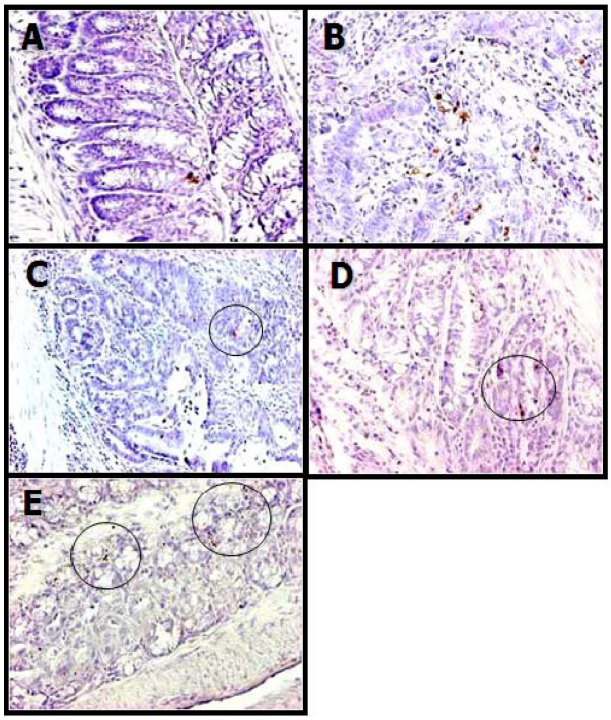

Fig. 8. TUNEL assay for apoptotic nuclei in distal colon sections of mice fed the low Fe diet. The TUNEL-positive cells were increased by treatment with AOM/DSS, which were further enhanced by co-administration of Se in a concentration dependent manner. vehicle control (A), AOM/DSS alone (positive control) (B), LFe + AOM/DSS + LSe (C), LFe + AOM/DSS + MSe (D), LFe + AOM/DSS + HSe (E). (× 100).

Discussion

The current study of AOM/DSS-induced colon carcinogenesis in the mouse model elucidated how the low Fe status influences colon carcinogenesis and how dietary Se affects the colon carcinogenesis in mice fed the low Fe diet. In our study, sodium selenate as dietary supplementation was used for Se source. The tumor incidences at three doses (0.02, 0.1, and 0.5 ppm Se in diet) were dependent on the concentration of Se in the mouse colon carcinogenesis model. The high dose (0.5 ppm) of Se suppressed the incidence of colonic cancer induced by AOM/DSS compared with low or medium dose of Se. These results were accordance with the cancer-chemopreventive effects of both inorganic (e.g., selenite and selenate) and organic forms of Se in humans and in animal models[21].

In present study, we established the Fe concentration of the low Fe diet to be 4.5 ppm Fe that is only about 10% of normal Fe diet (45 ppm Fe). The low Fe diet significantly lowered the Fe concentration in liver and the levels of mean

Table 4. Effect of Se on cell proliferative and apoptotic indices of mice fed the low Fe diet

Groups	Proliferative index (%)	Apoptotic index (%)
Vehicle	20.33 ± 2.91[a]	1.33 ± 0.67[a]
AOM/DSS alone (positive control)	64.33 ± 0.33[c]	8.33 ± 0.67[b]
LFe + AOM/ DSS + LSe	62.00 ± 4.08[c]	4.00 ± 0.55[c]
LFe + AOM/ DSS + MSe	46.25 ± 5.36[c]	5.00 ± 1.00[c]
LFe + AOM/ DSS + HSe	31.67 ± 1.42[b]	10.33 ± 2.03[d]

LFe: low Fe (4.5 ppm) diet, LSe: low Se (0.02 ppm) diet, MSe: medium Se (0.1 ppm) diet, HSe: high Se (0.5 ppm) diet. Data represented as mean ± SE. [abcd]Means in each column with different superscripts are significantly different ($p < 0.05$).

corpuscular volume and mean corpuscular hemoglobin, as indicators of microcytic hypochromic anemia in mice. Bhasin et al. [4] reported that a low Fe state reduces the tumor-promoting potential of benzoyl peroxide in DMBA-initiated murine skin. In our study the tumor incidence in low Fe diet group decreased by 5.8% compared to normal Fe diet group. Similarly, Ilsley et al. reported a significant decrease in both size and multiplicity of adenomas in the low Fe mice in the AOM-induced colon tumor model and suggest that Fe exerts its effect at the stage of tumor promotion, but is not sufficient to initiate tumor formation[22]. Lund et al. reported that Fe on the formation of reactive oxygen species (ROS) via a Fenton reaction is associated with changes in crypt cell proliferation in rat large intestine[23]. On the other hand, Soyars and Fischer reported that dietary Fe did not enhance oxidative stress, cell proliferation and ACF development in the colon of SD rats[24]. These results of studies cited above and the present study indicated that the effect of Fe on colon cancer is still controversial. In our results, the low Fe diet decreased tumor incidence. There were few reports on the relation between low Fe and colon cancer. Thus, further studies are required to elucidate the influence of low Fe mice on the colorectal carcinogenesis.

In the present study, the low Se diet increased the incidence of colonic cancer formation induced by AOM/DSS, whereas the high Se diet decreased the incidence compared with the positive control. The percentages of tumor incidence in mice fed the medium Se (positive control) and high Se diets were 10.8% and 13.3% lower than that found in the low Se diet group, respectively. Our results provide compelling evidence that supplement of Se in diet inhibits colon carcinogenesis. In this study, TUNEL assay and immunohistochemistry of PCNA were performed on the mucous membrane tissue of colon in order to confirm the association between Se and cell proliferation or apoptosis. On TUNEL assay, apoptotic positive cells were increased in the high Se diet group but decreased in low Se diet group. In the PCNA staining, the number of positively stained cells in the high Se diet group was lower than in the medium Se diet group or low Se diet group. From these results, Se induced the colon tumor cell apoptosis and inhibited cell proliferation. In previous studies, Se supplement can prevent tumorigenesis and decrease the incidence of cancer[25-26]. Also, a high Se was associated with a reduced prevalence of colorectal adenomas[14,26]. In previous study, cells are endowed with cytoprotective mechanisms (antioxidants, scavenging enzymes, repair processes) that act to counteract the effects of free radical production. Thus, the net effect of metal-induced free radicals on cellular function depends on the balance between radical production and the cytoprotective systems[27]. In addition, Fe may catalyze the production of proximate carcinogens (oxygen radicals) while Se may destroy them via antioxidant action of selenoproteins[28].

In other study, the anticarcinogenic action of Se depends on its chemical form, dosage and the nature of the carcinogenic agent whereas antimutagenic action of Se appears to be preventing the malignant transformation of normal cells and the activation of oncogenes[11]. Also, Schrauzer reported that cells adequately supplied with Se are less susceptible to the damaging effects of endogenously or exogenously generated oxygen radicals, which may attack cellular DNA, cause mutations and the oxidative activation of chemical carcinogens[11]. Although it is hypothesized that dietary Se in combination with low Fe can modulate a decrease of tumor incidence due to the mechanism by a decrease of reactive oxygen radicals induced by low Fe and destruction of reactive oxygen radicals.

As the results showed, the activity and protein expression of glutathione peroxidases (GPxs) in the low Se diet group was decreased while it was increased in the high Se diet. However, in low Fe mice, the activity of hepatic GPx in the medium Se diet group showed the higher than positive control (normal Fe group). Also, in our results, a reduction in MDA concentration in liver following Se supplementation might be associated with a reduction of lipid peroxidation and a possible protective effect on the events leading to tumor incidence. In previous study, expression of 14 oxidative stress-related molecules in both tumorous and non-tumorous tissues in 41 patients was examined by immunohistochemistry and Western blot analysis. GPx-1 and GPx-3 protein expression level was significantly decreased in human colon tumorous tissues, while MDA and 4-hydroxy-2-hexenal (4-HHE) levels were much higher than those in non-tumorous tissues[29]. Also, Ashokkumar and Sudhandiran reported that frequency of ACF, levels of MDA and hydroxyl radical were found to be increased, whereas catalase and GPx were

decreased in the plasma and colon of AOM-induced Balb/c mice[30]. Like the preceding report, the results showed that the activity of GPx, which is one of the antioxidants, is known to protect DNA and other cellular components from damage by oxygen radicals. Thus, in our study, the protective effects of Se seem to be primarily associated with GPx. Our results provide compelling evidence that supplement of the high Se diet causes enhancements of hepatic Se concentration and the GPx protein level, on the other hand, decrease of MDA levels and cellular damage by oxidative stress. Thus, the colon carcinogenesis was inhibited in part via the GPx activity based on the Se level.

In conclusion, these findings indicate that dietary Se might exert a modulating effect on colon carcinogenesis induced by AOM/DSS in mice fed the low Fe diet. The protective ability of Se at the low Fe status is considered to be mediated by modulating proliferation, apoptosis, GPx and MDA level and decreased the incidence rate of colon tumors in the AOM/DSS mouse model. These results indicate that dietary Se is a chemopreventive agent for colon carcinogenesis induced by AOM/DSS in male ICR mice. Also, further studies are required to elucidate Se-induced apoptosis mechanisms and the interaction between Fe and Se status on the colorectal carcinogenesis.

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