

Original Article

Effect of ascorbic acid and hemin on experimental colon carcinogenesis in mice

Ye-Seo Jeong^{1†}, Eun Young Kim^{2†}, Jae-Hwang Jeong³, Sang Yoon Nam¹, Beom Jun Lee^{1*}

¹College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Korea

²Korea Food Culture Promotion Association, Cheongju 28553, Korea

³Department of Biotechnology and Biomedicine, Chungbuk Province College, Cheongju 28160, Korea

Colon cancer is known as the third most widespread cancer in the world. The interaction of heme-iron and ascorbic acid (AA) in colon carcinogenesis is not evident. Hemin (ferric chloride heme) is an iron-containing porphyrin with chlorine that can be formed from a heme group. The purpose of this study was to investigate the protective effect of AA on the formation of pre-neoplastic lesions induced by azoxymethane (AOM)/dextran sodium sulfate (DSS) plus hemin in mice. Forty-five ICR male mice were divided into three experimental groups; AOM/DSS treatment (control group), hemin (2 g hemin/kg of b.w.), hemin + AA (1.0% in drinking water). The mice had three s.c. injections (0–2nd weeks of the experiment) of AOM (10 mg/kg b.w.) weekly and 2% DSS as drinking water for the next one week and the animals fed on AIN-76A purified rodent diet for 6 weeks. The numbers of aberrant crypt foci (ACF) and aberrant crypts (ACs) in colonic mucosa were counted after methylene blue staining. Lipid peroxidation in feces was measured by the thiobarbituric acid-reactive substances (TBARS) assay. The numbers of ACF and ACs per colon significantly increased in Hemin group compared to the control group. However, the numbers of ACF and ACs per colon notably decreased in hemin + AA group compared to the control group or hemin group ($p < 0.05$). In feces, the TBARS value of hemin group was higher than the control group ($p < 0.01$). The TBARS value of hemin + AA group was slightly decreased compared to Hemin group. These results indicate that hemin can promote the experimental colon carcinogenesis in ICR mice. On the other hand, additional supplement of AA via drinking water has a protective effect against the colon carcinogenesis.

The related mechanisms need to be illustrated by further studies in future.

Key words: colon cancer, ascorbic acid, aberrant crypt foci, azoxymethane, hemin

Introduction

Colon cancer is the third most widespread cancer with high morbidity and mortality, and the fourth common reason of cancer deaths in the world [1]. In many publications, colon and rectal cancer have been researched collectively and the term, colorectal cancer (CRC), is used [1]. Diet would firmly affect CRC risk, and modifications in foods habits might lessen up to 70% of this cancer risk [2]. Published in 1997, the World Cancer Research Fund authoritative expert report states: “evidence shows that red meat probably increases risk and processed meat possibly increases risk of CRC” [3].

Since 2000, three meta-analyses suggested that total meat consumption has nothing to do with risk, but that red meat consumption is an important risk factor [4]. One of the leading hypotheses that have been studied experimentally is that heme-iron in red meat can trigger carcinogenesis because it arises cell proliferation in the colonic mucosa, through lipoperoxidation of feces [5]. Sesink insisted that the oxidative form (Fe^{3+}) of heme, hemin represents that red meat intake has relevance to the risk of CRC [6]. Heme iron is thought to catalyze the endogenous formation of N-nitroso compounds in the colon, many of which are known carcinogens [7]. Therefore, excessive heme-iron can contribute to cause cancer in the

[†]These authors contributed equally to this work.

*Corresponding author: Beom Jun Lee

College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Korea
Tel: +82-43-261-3357, Fax: +82-43-271-3246, E-mail: beomjun@cbu.ac.kr

colon.

Azoxymethane (AOM) and deodesyl sodium sulfate (DSS) are used to start the initiation of CRC by causing aberrant crypt foci (ACF). First reported in 1996, AOM/DSS mouse model was proven as an outstanding model in faithfully mimicking of the pathological changes of “normal-ACF-adenoma-carcinoma” development as observed in human CRC, and hence was widely used in studies of colorectal carcinogenesis and chemopreventive intervention [8, 9]. ACF have emerged as a putative precursor to colorectal adenoma, and have been suggested to be a potentially useful biomarker for CRC [10]. Dietary heme iron (hemin) was found to cause similar colonic changes as meat-based diets in AOM-treated rats [11], and changes in gene expression linked to cancer and proliferation were detected in colon scrapings of mice after only 4 days of heme iron (hemin) administration [12]. In addition, Hemin and hemoglobin increase the number of AOM-induced ACF which are the previous stage colon cancer of rat [13].

Meanwhile, epidemiologic evidence suggests that vitamin C-rich foods play a protective role against development of cancer [14]. Plasma concentrations of ascorbic acid (AA) have been shown to be inversely associated with cancer risk [14]. The fact that AA is required to maintain full function of an array of enzymes indicates that optimizing intake would optimize metabolism as well as prevent cancer [15]. Although there are numerous studies about heme-iron effect on CRC model and influence of AA with iron, the interaction between AA and hemin in CRC is not evident. In this research, the protective effect of AA was investigated on pre-neoplastic lesions arose from AOM/DSS with hemin in mice.

Materials and Methods

Materials

AOM was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dextran sodium sulfate (DSS) whose molecular weight is 36,000–50,000 was obtained from MP Bio-medical (Irvine, CA, USA). Hemin ($\geq 97.0\%$) which is powder forms was obtained from Sigma-Aldrich. L-ascorbic acid was obtained from Sigma-Aldrich.

Animals and experimental diets

Male ICR mice (4 weeks old) were obtained from Orientbio Laboratory Animal Seongnam, Korea) and housed in isolating polycarbonate cage (5 mice/cage). The temperature and relative humidity were maintained at $20 \pm 2^\circ\text{C}$ and $50 \pm 20\%$. Light and dark cycles were at 12 h each and the intensity of illumination was maintained at

150–300 lux. The AIN-76A purified diet purchased from Central Animal Laboratory (Seoul, Korea) was given to the mice. During the experimental periods, diets and litter were all used after sterilization and the animal experiment was conducted in compliance with “Guide for care and use of Laboratory animals” of Chungbuk National University. During the experimental period, weekly body weight and water consumption were recorded. The experiment was approved by Chungbuk National University Institutional Animal Care and Use Committees with the approval number, CBNUA-81A-17-01.

Experimental design

After acclimation for 1 week, five-week old mice was divided into 3 groups (15 mice/group) including (1) carboxymethylcellulose (CMC): control group, (2) CMC + hemin group, (3) CMC + hemin + 1.0% AA dissolved in distilled water group. AIN-76A purified diet was fed to the three groups. Hemin (2 g/kg B.W.) mixed with 1.0% CMC was orally administrated to each group. Also, the mice were subcutaneously treated with AOM (10 mg/kg B.W.) three times at the 0, 1st, and 2nd weeks of the experimental period to induce the formation of pre-neoplastic lesion in colon. From the next day, drinking water containing 2.0% DSS was provided for 7 days and the total experimental period is 6 weeks (Fig. 1).

Total number of aberrant crypt and aberrant crypt foci count

At 6 weeks after the experiment, all mice were sacrificed. After performing cervical dislocation, the colons were harvested and flushed with 0.9% NaCl solution and finally fixed in 10% neutral phosphate buffered formalin. The formalin-fixed colonic tissues were stained in 0.2% methylene blue solution for 30 sec. The numbers of ACF and aberrant crypts (ACs) were counted under a microscope (40–100 \times). ACF were identified via the following morphological characteristics: 1) enlarged and elevated crypts compared to normal mucosa and 2) increased pericryptal space and irregular lumen [16]. The total number of ACF was recorded as number/colon (Fig. 2).

Feces preparation

Feces sample was collected from 5 mice/one cage. One sample thus corresponded to the pooled feces from five mice. Feces were prepared by reconstituting freeze-dried feces by adding 1 mL of distilled water to 0.3 g of freeze-dried feces. Samples were incubated at 37°C for 1 h and were thoroughly mixed during the incubation time. They were then centrifuged for 15 min at 20,000 g, and supernatants were collected and stored at -20°C until use.

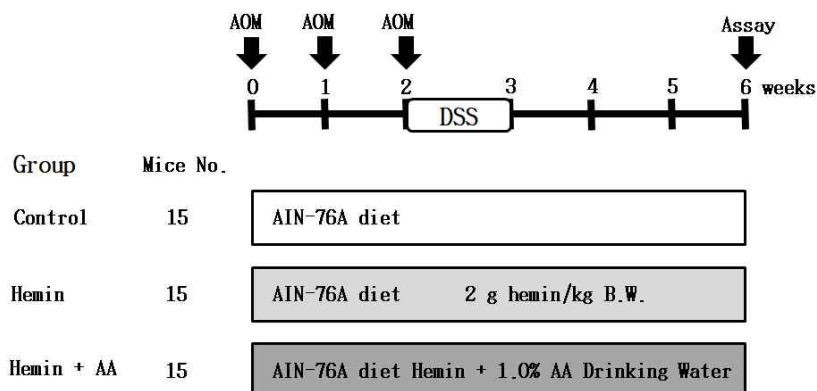


Fig. 1. Experimental design for induction of colon carcinogenesis and treatment scheme in mice. AOM, azoxymethane (10 mg/kg body weight in saline, S.C., weekly 3 times). DSS, dextran sodium sulfate (2.0% in drinking water for a week). Hemin (2 g/kg B.W. in carboxymethyl cellulose. P.O., daily). AA, ascorbic acid (1.0% in drinking water for 6 weeks).

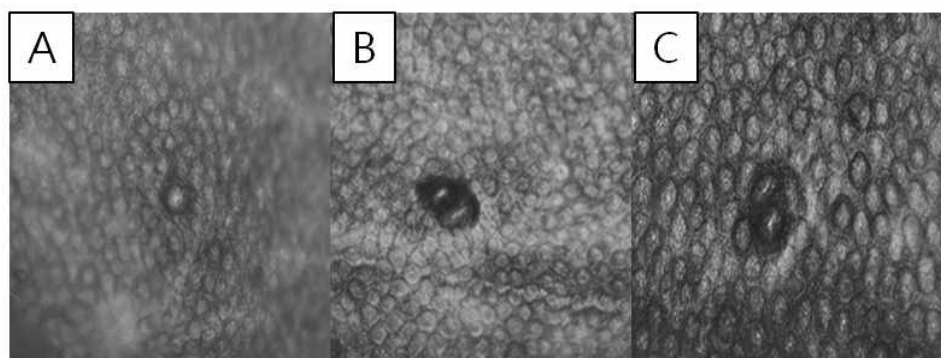


Fig. 2. Colonic aberrant crypt focus stained with methylene blue after treatment of AOM/DSS in mice. (A) AC (Aberrant crypt) focus with a crypt. (B) AC focus with 2 crypts. (C) AC focus with 3 crypts. Magnification: $\times 100$. AOM, azoxymethane; DSS, dextran sodium sulfate.

Thiobarbituric acid reactive substances (TBARS) assay

TBARS assay was conducted for determination of malondialdehyde of which is a by-product of lipid peroxidation in feces. At first, collected sample 100 μL , 8.1% sodium dodecyl sulfate (SDS) solution 100 μL , 20% acetic acid solution 200 μL were mixed together and 0.75% 2-thiobarbituric acid solution 100 μL were added lastly and they were vortexed. Then, the incubation at 95°C was conducted for 1 hr and the centrifuge process was conducted at 4°C, 1,600 g for 10 min. At last, they were put in the 96-well plate and the optical density of the supernatant liquid was measured at 530 nm. Standards were used MDA concentrations at 0, 100, 0.625, 1.25, 2.5, 5, 10, 25, and 50 mM.

Histopathological examination

The tissues fixed in 10% neutral buffered formalin were

paraffin-embedded, cut into 5- μm sections, and stained with hematoxylin and eosin for histopathological examination under a light microscope (Olympus, Tokyo, Japan) ($\times 100$).

Statistical analysis

Data were expressed as the means \pm SD. Data were analyzed with the one-way ANOVA followed by Tukey's posttest and Kruskal-wallis test. The significant differences were determined at the level of $p < 0.05$ or $p < 0.01$.

Results

Changes in body weights

The mice body weights of all experimental groups were increased with the passage of time. During the 6-week experimental period, hemin + AA group had lower body weights compared with the control groups and hemin

group without a significance (Fig. 3).

Total number of aberrant crypt (AC) and aberrant crypt foci (ACF) count

Total number of ACs in hemin group (31.2 AC/colon) were significantly increased compared to control group (14.9 AC/colon) ($p < 0.01$). Total number of ACs in hemin + AA group (15.7 AC/colon) were significantly decreased compared to hemin group ($p < 0.01$). But there was no a significant difference between control group and hemin + AA group (Fig. 4).

Total number of ACF in hemin group (23.7 ACF/colon)

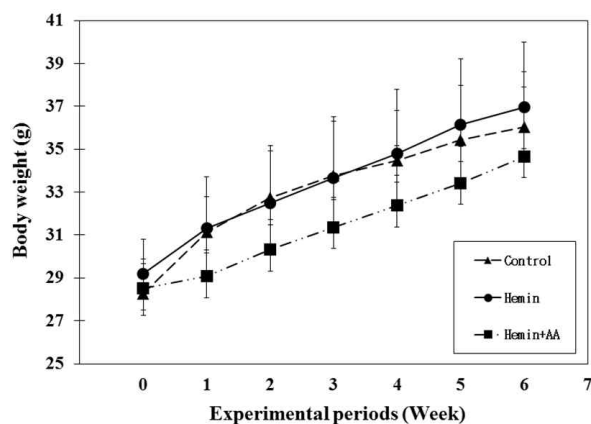


Fig. 3. Change in the body weights in mice of experimental groups. Each bar means the mean \pm SD ($n = 15$). There was no significant difference among experimental groups. AA, ascorbic acid.

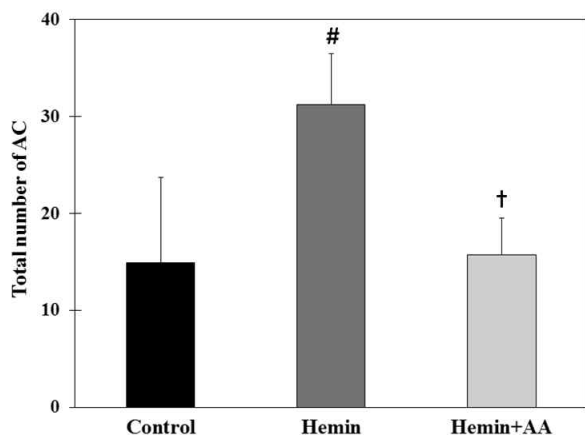


Fig. 4. Effect of hemin and ascorbic acid on formation of aberrant crypt in the colon of mice. Each bar means the mean \pm SD ($n = 15$). #Significantly different from control group ($p < 0.01$). †Significantly different from hemin group ($p < 0.01$). AA, ascorbic acids.

were significantly increased compared to control group (11.5 ACF/colon) ($p < 0.01$). Total number of ACF in hemin + AA group (11.29 ACF/colon) decreased compared to hemin group ($p < 0.01$). There was no significant difference in the number of ACs between control and hemin + AA group (Fig. 5).

TBARS values in feces

Lipid peroxidation was thus measured in fecal water by the TBARS assay. The TBARS values were the lowest in AOM/DSS control group among experimental groups (Fig. 6). Hemin treatment significantly increased the TBARS value compared with the control ($p < 0.01$), but AA supplementation by drinking water significantly decreased the TBARS value compared with the hemin group ($p < 0.01$) (Fig. 6).

Histopathology

AOM treatment showed earliest dysplastic colon epithelium prior to tumor development. Hemin group showed a thicker epithelium and inflammatory infiltrates in colon mucosa. Hemin + AA group ameliorate the histopathological changes (Fig. 7).

Discussion

The colon is the most prevalent site for neoplasms of the digestive tract, and CRC accounts for the second highest percentage of cancer mortality rate in males dwelling in developed countries [17]. It does not seem that colon cancer is a consequence of aging, but it is

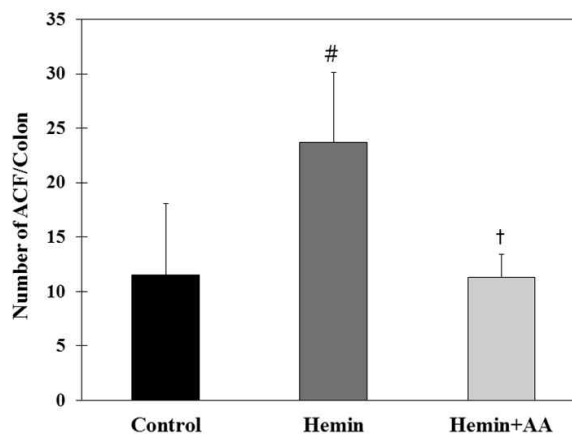


Fig. 5. Effect of hemin and ascorbic acid on formation of aberrant crypt foci in the colon of mice. Each bar means the mean \pm SD ($n = 15$). # Significantly different from control group ($p < 0.01$). † Significantly different from hemin group ($p < 0.01$). AA, ascorbic acids.

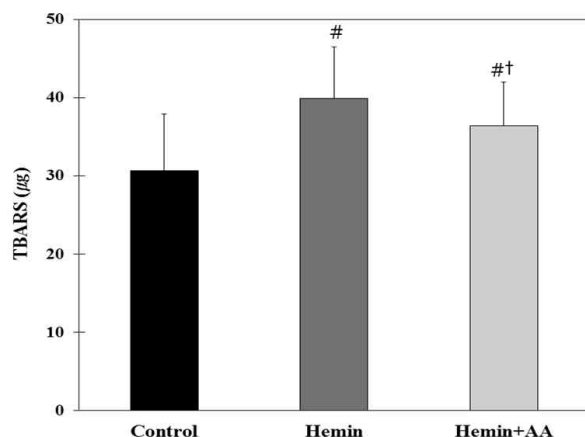


Fig. 6. Effect of hemin and ascorbic acid on lipid peroxidation in fecal water as measured by TBARS assay. Each bar means the mean \pm SD ($n = 3$). [#] Significantly different from control group ($p < 0.01$). [†] Significantly different from hemin group ($p < 0.05$). AA, ascorbic acids; TBARS, thiobarbituric acid-reactive substances.

basically related to dietary habits. The consumption of red and processed meat increases the risk of CRC, and this assertion is discovered to be associated firmly with the consumption of dietary iron [18]. Dietary heme-iron significantly increased the colon malignancies in mice at the aberrant crypt stage. This result advocates the hypothesis that heme can explain the correlation between red meat consumption and colon cancer risk [19]. Certainly, this study showed that 1) heme as hemin increased the number of AOM/DSS-induced ACF in the colon of mice, 2) hemin effect was associated with lipid peroxidation in fecal water, 3) hemin effect was inhibited by the consumption of AA in drinking water. The effect of hemin and AA, as an antioxidant, is discussed below.

Hemin was used to analyze the effect of heme from red meat because meat hemoproteins are digested in the sto-

mach and the small bowel, and provide free heme to the colon [20]. We investigated the influence of AA on the formation of colonic ACF induced by AOM/DSS in ICR mice. AA (1.0%) was administered via drinking water for 6 weeks. In the present study, hemin strikingly increased the number of AOM/DSS-induced ACF and ACs. Meanwhile, 1.0% AA treatment significantly decreased the number of total ACF and ACs compared to hemin group. ACF are putative pre-neoplastic lesions that have been detected in human colon cancer and experimental animals treated with chemical carcinogens. ACF can be identified microscopically on the surface of the whole mount colon mucosa after methylene blue staining. They are distinguished from normal crypts by their larger size, darker staining, and increased pericryptal space [21]. In this study, the number of ACF was counted and the result shows that orally administered hemin surely negatively affected on the formation of colonic pre-neoplastic lesions in mice. On the other hand, the result in hemin + AA group, as it showed lesser numbers of ACF and ACs than those of hemin group, supports that AA was definitely effective on defending the negative effect of hemin on colonic pre-neoplastic lesions in mice. These results suggest that AA supplementation via drinking water can inhibit the development of experimentally induced pre-neoplastic lesions. The role of AA is likely to involve several functions. It acts as an electron donor and as a reducing agent which allows it to have a cytotoxic effect. The pharmacologic doses of AA decrease the proliferation of colon cancer cells and induce their apoptosis and necrosis [22].

One attribute of red meat that has been studied extensively to determine its potential contribution to colon cancer development is iron. Most studies evaluate iron impacts using hemoglobin or other heme compounds as a surrogate for the heme-containing proteins in meat. Heme catalyzes the formation of lipid oxidation end-products,

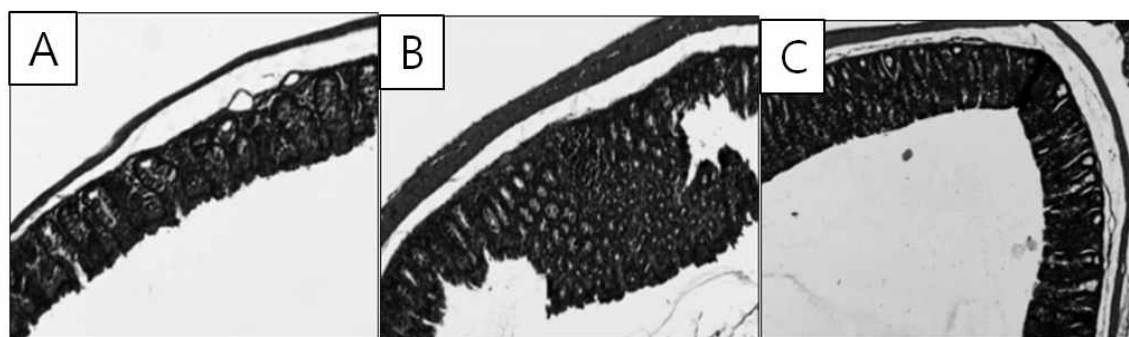


Fig. 7. Histopathology of colonic epithelium in experimental groups as stained by H&E ($\times 100$). (A) Control group, (B) Hemin group, (C) Hemin + ascorbic acid.

which may explain the promoting effects of red and processed meats on CRC [23]. In the present study, lipid peroxidation in feces was evaluated by the TBARS assay. TBARS results from lipid peroxidation of polyunsaturated fatty acids and is a marker for oxidative stress [23]. In this study, the AOM/DSS control group showed the lowest level of TBARS but orally-administration of hemin increased the TBARS value in the fecal water. Fecal water-soluble components derived from hemin fed rats led to higher lysis of erythrocytes, suggesting hemin promoted a cytotoxic environment [24]. The resulting epithelial surface injury induced by hemin or its metabolites led to an increase in cell proliferation and a reduction in apoptosis to maintain epithelial barrier function [24]. In this study, there is a tendency to decrease the formation of lipid oxidation end-products when AA was supplied via drinking water, in combination with hemin treatment. Therefore, although the level of TBARS in hemin + AA group was not as low as that of control group, it is possible to say that AA suppressed the hemin-induced cytolytic activity of fecal water.

In conclusion, when hemin was administered to mice, there is a tendency to increase the formation of ACs, ACF and lipid oxidation end-products. However, the treatment with AA via drinking water likely decreased the increased formation of pre-neoplastic lesions in mice. From these results, it is possible to conclude that AA has a defense effect on meat-promoted colon cancer in human and animals. Further research might be required to prove the detailed mechanisms.

Conflict of Interest

The authors declare that they have no competing interests.

Acknowledgements

This work was financially supported by the Research Year of Chungbuk National University in 2019.

ORCID

Ye-Seo Jeong, <https://orcid.org/0000-0003-0372-0137>
 Eun Young Kim, <https://orcid.org/0000-0002-9972-664X>
 Jae-Hwang Jeong, <https://orcid.org/0000-0003-3920-3799>
 Sang Yoon Nam, <https://orcid.org/0000-0001-7576-6543>
 Beom Jun Lee, <https://orcid.org/0000-0002-7013-8086>

Ethics Approval

The number of IACUC approval is CBNUA-81A-17-01.

References

1. Bastide NM, Pierre FH, Corpet DE. Heme iron from meat and risk of colorectal cancer: a meta-analysis and a review of the mechanisms involved. *Cancer Prev Res (Phila)* 2011; 4:177-184.
2. Cummings JH, Bingham SA. Diet and the prevention of cancer. *BMJ* 1998;317:1636.
3. Glade MJ. Food, nutrition, and the prevention of cancer: a global perspective. American Institute for Cancer Research/World Cancer Research Fund, American Institute for Cancer Research, 1997. *Nutrition* 1999;15:523-526.
4. Norat T, Lukanova A, Ferrari P, Riboli E. Meat consumption and colorectal cancer risk: dose-response meta-analysis of epidemiological studies. *Int J Cancer* 2002;98:241-256.
5. Santarelli RL, Pierre F, Corpet DE. Processed meat and colorectal cancer: a review of epidemiologic and experimental evidence. *Nutr Cancer* 2008;60:131-144.
6. Sesink AL, Termont DS, Kleibeuker JH, Van der Meer R. Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. *Cancer Res* 1999;59:5704-5709.
7. Cross AJ, Pollock JR, Bingham SA. Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. *Cancer Res* 2003;63:2358-2360.
8. Okayasu I, Ohkusa T, Kajiura K, Kanno J, Sakamoto S. Promotion of colorectal neoplasia in experimental murine ulcerative colitis. *Gut* 1996;39:87-92.
9. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996;87:159-170.
10. Fujii T, Sakai E, Takahashi H, Yamada E, Ohkubo H, Higurashi T, Nakajima A. The distribution of human rectal aberrant crypt foci and criteria for defining the counting area. *Oncol Lett* 2017;13:4501-4504.
11. Pierre F, Freeman A, Taché S, Van der Meer R, Corpet DE. Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons. *J Nutr* 2004;134:2711-2716.
12. Ijssennagger N, Rijnierse A, de Wit NJ, Boekschoten MV, Dekker J, Schonewille A, Müller M, van der Meer R. Dietary heme induces acute oxidative stress, but delayed cytotoxicity and compensatory hyperproliferation in mouse colon. *Carcinogenesis* 2013;34:1628-1635.
13. Pierre F, Taché S, Petit CR, Van der Meer R, Corpet DE. Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats. *Carcinogenesis* 2003;24:1683-1690.
14. Patterson RE, White E, Kristal AR, Neuhauser ML, Potter

- JD. Vitamin supplements and cancer risk: the epidemiologic evidence. *Cancer Causes Control* 1997;8:786-802.
15. Levine M, Eck P. Vitamin C: working on the x-axis. *Am J Clin Nutr* 2009;90:1121-1123.
16. McLellan EA, Bird RP. Aberrant crypts: potential preneoplastic lesions in the murine colon. *Cancer Res* 1988;48:6187-6192.
17. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011;61:69-90.
18. Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst* 1981;66:1192-1308.
19. Sesink AL, Termont DS, Kleibeuker JH, Van der Meer R. Red meat and colon cancer: dietary haem-induced colonic cytotoxicity and epithelial hyperproliferation are inhibited by calcium. *Carcinogenesis* 2001;22:1653-1659.
20. Vaghefi N, Nedjaoum F, Guillochon D, Bureau F, Arhan P, Bouglé D. Influence of the extent of hemoglobin hydrolysis on the digestive absorption of heme iron. An *in vitro* study. *J Agric Food Chem* 2002;50:4969-4973.
21. Bird RP. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett* 1987;37:147-151.
22. Halabi IE, Bejjany R, Nasr R, Mukherji D, Temraz S, Nassar FJ, Darsa HE, Shamseddine A. Ascorbic acid in colon cancer: from the basic to the clinical applications. *Int J Mol Sci* 2018;19:2752.
23. Sawa T, Akaike T, Kida K, Fukushima Y, Takagi K, Maeda H. Lipid peroxyl radicals from oxidized oils and heme-iron: implication of a high-fat diet in colon carcinogenesis. *Cancer Epidemiol Biomarkers Prev* 1998;7:1007-1012.
24. Turner ND, Lloyd SK. Association between red meat consumption and colon cancer: a systematic review of experimental results. *Exp Biol Med (Maywood)* 2017;242:813-839.