

High-fat Diet Accelerates Intestinal Tumorigenesis Through Disrupting Intestinal Cell Membrane Integrity

ORIGINAL
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Background: Excess energy supply induces chronic low-grade inflammation in association with oxidative stress in various tissues including intestinal epithelium. The objective of this study was to investigate the effect of high-fat diet (HFD) on intestinal cell membrane integrity and intestinal tumorigenesis in *Apc^{Min/+}* mice.

Methods: Mice were fed with either normal diet (ND) or HFD for 12 weeks. The number of intestinal tumors were counted and biomarkers of endotoxemia, oxidative stress, and inflammation were determined. Changes in intestinal integrity was measured by fluorescein isothiocyanate (FITC)-dextran penetration and membrane gap junction protein expression.

Results: HFD group had significantly higher number of tumors compared to ND group ($P < 0.05$). Blood total antioxidant capacity was lower in HFD group, while colonic 8-hydroxy-2'-deoxyguanosine level, a marker of oxidative damage, was higher in HFD group compared to that of ND group ($P < 0.05$). The penetration of FITC-dextran was substantially increased in HFD group ($P < 0.05$) while the expressions of membrane gap junction proteins including zonula occludens-1, claudin-1, and occludin were lower in HFD group ($P < 0.05$) compared to those in ND group. Serum concentration of lipopolysaccharide (LPS) receptor (CD14) and colonic toll-like receptor 4 (a LPS receptor) mRNA expression were significantly higher in HFD group than in ND group ($P < 0.05$), suggesting that significant endotoxemia may occur in HFD group due to the increased membrane permeability. Serum interleukin-6 concentration and myeloperoxidase activity were also higher in HFD group compared to those of ND group ($P < 0.05$).

Conclusions: HFD increases oxidative stress disrupting intestinal gap junction proteins, thereby accelerating membrane permeability endotoxemia, inflammation, and intestinal tumorigenesis.

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Key Words: *Apc*, Mice, Colon neoplasms, High-fat diet, Permeability, Oxidative stress

INTRODUCTION

Colon cancer is the fourth leading cause of cancer deaths worldwide.¹ Epidemiological studies provided evidence that environmental factors are the prime importance in the etiology of colon cancer.² One of the most important risk factors for colon cancer is body fatness which is often resulted from imbalance between energy intake and energy expenditure.^{3,4} Therefore, excess dietary fat intake in association with body fat deposition

has been regarded as a convincing risk factor for tumorigenesis in the colon. In fact, a chronic fat-rich diet, a typical high energy diet, could results in increased endotoxemia which is a triggering factor for inflammatory responses promoting carcinogenesis.⁵

Excess reactive oxygen species (ROS) generation in the obese state results in increased oxidative damage which accelerate inflammatory responses.⁶ Several human studies have shown that obese subjects exhibit lower systemic antioxidant defense activity than normal weight counterparts. Serum 8-hydroxy-2'-de-

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oxyguanosine (8-OHdG) concentration, a marker of oxidative damage, has also been found higher in pre-diabetic subjects.⁷⁻⁹ Increased oxidative stress is implicated in carcinogenesis through DNA, protein, and lipid oxidation, and modulation of cellular signaling.¹⁰⁻¹² Especially, intestinal epithelial cells grow rapidly and have a high metabolic rate which makes them extremely vulnerable to oxidative DNA damage.¹³ Genetic alterations as well as signaling pathways to deal with the oxidative burden in epithelium are well known to be involved in the carcinogenesis.

Several lines of evidence indicate that oxidative stress also disrupts epithelial tight junctions (TJs) and increases the membrane permeability. In obese patients and animal models of obesity, intestinal permeability is shown to be increased possibly due to impairment of TJs.¹⁴⁻¹⁶ Although the causes and mechanisms of disruptions in TJs are not fully understood, alterations in microbiota composition and chronic low-grade inflammation may have been suggested to be linked to intestinal membrane integrity.¹⁷⁻¹⁹ The increase of intestinal permeability causes the development of metabolic endotoxemia with increased lipopolysaccharide (LPS) translocation into the systemic circulation.²⁰

Apc^{Min/+} mice exhibit a germ-line nonsense mutation at codon 850 of adenomatous polyposis coli (*APC*) gene and spontaneously develop multiple polyps in the small and large intestines at the age of 10 to 12 weeks.²¹ Therefore, *Apc*^{Min/+} mice are considered to be a useful animal model to study human familial adenomatous polyposis and colorectal cancers.

Although several studies have investigated possible link between systemic fat deposition and oxidative stress or inflammatory responses, few studies have been reported that the involvement of transepithelial colon permeability in high-fat diet (HFD) induced colon tumorigenesis. Therefore, in this study, we assessed the effects of HFD on oxidative damage, local inflammatory events, and intestinal integrity in association with intestinal tumorigenesis in *Apc*^{Min/+} mice.

MATERIALS AND METHODS

1. Animals and study design

The inbred mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The *Apc*^{Min/+} pedigree was maintained by mating *Apc*^{+/+} females with *Apc*^{Min/+} males, and procedures to secure inbreeding were followed. The *Apc*^{Min/+} mice were identified by allele-specific PCR on DNA isolated from tail. All animals were housed in wire cages (4-5 mice/cage) with free access to drinking water and basal diet CE-2 (CLEA Japan Inc., Tokyo, Japan) under controlled conditions of humidity (50% ±

10%), lighting (12-hour light/dark cycle), and temperature (23°C ± 2°C), which were maintained until termination of the study. The composition of experimental diet was based on a modified AIN-93G diet as shown in Table 1. The fat sources of normal diet (ND, 15% of fat calories) and HFD (45% of fat calories) were based on corn oil and lard. Mice were fed either ND or HFD for 12 weeks. Weight gain and food intake were recorded twice a week. All animals were sacrificed at the end of the study period using Zoletil and Rompun as an anesthetic. The colons were removed and rinsed in ice-cold normal saline. They were laid flat on a glass plate, and the mucosa was scraped with a slide glass. Epididymal fat pad and liver were collected immediately, rinsed in ice-cold normal saline, and weighed. Blood was collected from the inferior vena cava into EDTA tubes and centrifuged at 4,000 rpm for 20 minutes. All samples were stored at -80°C until assayed. An animal protocol approved by the Institutional Animal Care and Use Committee of Sookmyung Women's University was used for all animal experiments.

2. Tumor scoring

At sacrifice, all organs were removed, and the small and large intestines were cut open along their longitudinal axis. The small intestine and colon were then spread flat on a microscope slide and the number of tumors was determined with an inverse light microscope with a magnification of 1 × 10.

Table 1. Composition of the experimental diets^a

Variable	ND	HFD
Macronutrient composition		
Carbohydrate (% of energy)	65.700	35.700
Protein (% of energy)	19.300	19.300
Fat (% of energy)	15.000	45.000
Ingredient (g/kg)		
Cornstarch	404.000	266.500
Dextrin	134.200	88.500
Sucrose	101.600	67.100
Fiber	50.000	50.000
Casein	198.000	240.400
Corn oil	12.440	45.360
Lard	49.760	181.440
Mineral mix ^b	34.600	42.100
Vitamin mix ^b	9.900	12.000
L-cysteine	3.000	3.700
Choline bitartrate	2.500	3.100
Tert-butylhydroquinone	0.014	0.017
Total calories/100 g	372.800	452.800

ND, normal diet; HFD, high-fat diet. ^aDiets were prepared according to the AIN-93 G diet with slight modifications. ^bMineral mixture and vitamin mixture were prepared according to AIN-93 G diet.

3. Total antioxidant capacity

Total antioxidant activity was determined by the ferric reducing antioxidant power (FRAP) assay. The FRAP reagent was prepared from 10 mmol/L 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ) solution in HCl 40 mmol/L plus FeCl₃ (20 mmol/L) and acetate buffer (0.3 mol/L, pH 3.6) in a 1:1:10 ratio. Freshly prepared FRAP reagent was warmed at 37°C for 5 minutes. Serum sample or standard (50 µL) was mixed with 1.5 mL of FRAP reagent in a test tube and incubated at 37°C for 10 minutes. Then, the absorbance of the colored products (ferrous TPTZ complex) was measured at 593 nm and compared to the blank.

4. Colonic 8-hydroxy-2'-deoxyguanosine concentration

Colons were homogenized and DNA was extracted using DNA extraction kit (Nirthwest Life Science Specialities, Vancouver, BC, Canada) following the manufacture's instruction. Extracted DNA (200 µg) was dissolved in 135 µL of water, and 15 µL of 200 mM sodium acetate and 6 units of nuclease P1 were added to the DNA solution and incubated for 30 to 60 minutes at 37°C. Tris-HCl buffer (1 M, pH 7.4) and 2 unit of alkaline phosphatase were added and incubated for 30 to 60 minutes at 37°C. After filtering the solution, the supernatants were used for the assay.

5. Determination of intestinal permeability

This measure is based on the intestinal permeability to 4,000-Da fluorescent-dextran (Sigma-Aldrich, St. Louis, MO, USA) as described previously. Briefly, 6-hour fasted mice were injected with fluorescein isothiocyanate (FITC)-dextran by gavage (600 mg/kg body weight, 125 mg/mL) and 120 µL blood was collected from the heart 1 hour after the gavage. The blood was centrifuged at 4°C, 4,000 rpm, for 3 minutes. Plasma was diluted in an equal volume of PBS (pH 7.4) and analyzed for FITC-dextran concentration with a fluorescence spectrophotometer at the excitation wavelength of 485 nm and the emission wavelength of 535 nm. Standard curve for calculating the FITC-dextran concentration in the samples were obtained by diluting FITC-dextran in non-treated plasma diluted with PBS (1:2).

6. Western blot analysis

For the immunoblotting analysis of zonula occludens-1 (ZO-1), claudin-1, and occludin, sample proteins (30 µg) were electrophoresed through 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The transferred membrane was blocked using 2% skim milk to inhibit non-specific proteins, and treated with primary antibodies

against ZO-1 (Invitrogen, Carlsbad, CA, USA), claudin-1 (Invitrogen), occludin (Invitrogen), and β-actin (Sigma-Aldrich). Anti-mouse immunoglobulin G conjugated with alkaline phosphatase was used as the secondary antibody. Each protein band was then confirmed and quantified using an enhanced chemiluminescence system (Amersham, Arlington Heights, IL, USA). The integrity of band was quantified by Versa Doc Quantity one program (BioRad, Mississauga, ON, Canada).

7. Determination of colonic toll-like receptor 4 mRNA expression

Total RNA was isolated from the mouse colon using Trizol reagent (Invitrogen) following the manufacture's recommendation. Reverse transcription of total RNA samples and PCR were accomplished using the premix RNA PCR kit (Invitrogen) according to the manufacturer's instruction. PCR primers were designed using nucleotide sequence for mouse toll-like receptor 4 (TLR4; Bioneer, Daejeon, Korea). The following primers were used: TLR4, forward 5'-AAT TCC TGC AGT GGG TCA AG-3' and reverse 5'-AGG CGA TAC AAT TCC ACC TG-3'; GAPDH, forward 5'-ACC TCT ATG CCA ACA CAG TGC-3' and reverse 5'-CTC ATC GTA CTC CTG CTT GC-3'. The cycling conditions were 3 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 1 minute at 72°C, and 10 minutes at 72°C. PCR products were confirmed by 2% agarose gel electrophoresis and visualized by UV transillumination (Bio-Rad Laboratories Inc., Hercules, CA, USA). For quantitative analysis, Versa Doc Image analyzer (Amersham Biosciences, Piscataway, NJ, USA) was used. All signals were normalized to the mRNA levels of the housekeeping gene β-actin and expressed as ratio.

8. Biochemical measurement

Serum CD14 level was determined using commercially available ELISA kit (Cell Sciences Inc., Canton, MA, USA) according to the manufacturer's instruction. Interleukin (IL)-6 level was evaluated in plasma using commercially available ELISA kit (BD Bioscience Co., Franklin Lakes, NJ, USA) according to the manufacturer's instruction. Plasma myeloperoxidase (MPO) activity was determined using commercially available ELISA kit (EIAab Science Co., Wuhan, China) according to the manufacturer's instruction.

9. Statistical analysis

Statistical analysis was performed by using the SAS package (release 8.01; SAS Institute, Cary, NC, USA). All data from the experiment were expressed as mean ± SD. Data were analyzed by

Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

1. Effect of high-fat diet on body weight, epididymal fat weight, liver weight, and tumor number

The body weight of HFD group was significantly higher than that of ND group between experimental week 2 and 5; however, the difference disappeared thereafter possibly due to faster

tumor development in HFD group (Fig. 1A). No difference was found in liver weight and epididymal fat weight (Fig. 1B and 1C). However, the average tumor number was significantly higher in HFD mice compared to that of the ND mice ($P < 0.05$; Fig. 1D).

2. Effect of high-fat diet on blood total antioxidant capacity and colonic 8-hydroxy-2'-deoxyguanosine level

The total antioxidant capacity (TAC) is an indicator of oxidative stress. The level of 8-OHdG is one of the predominant forms of

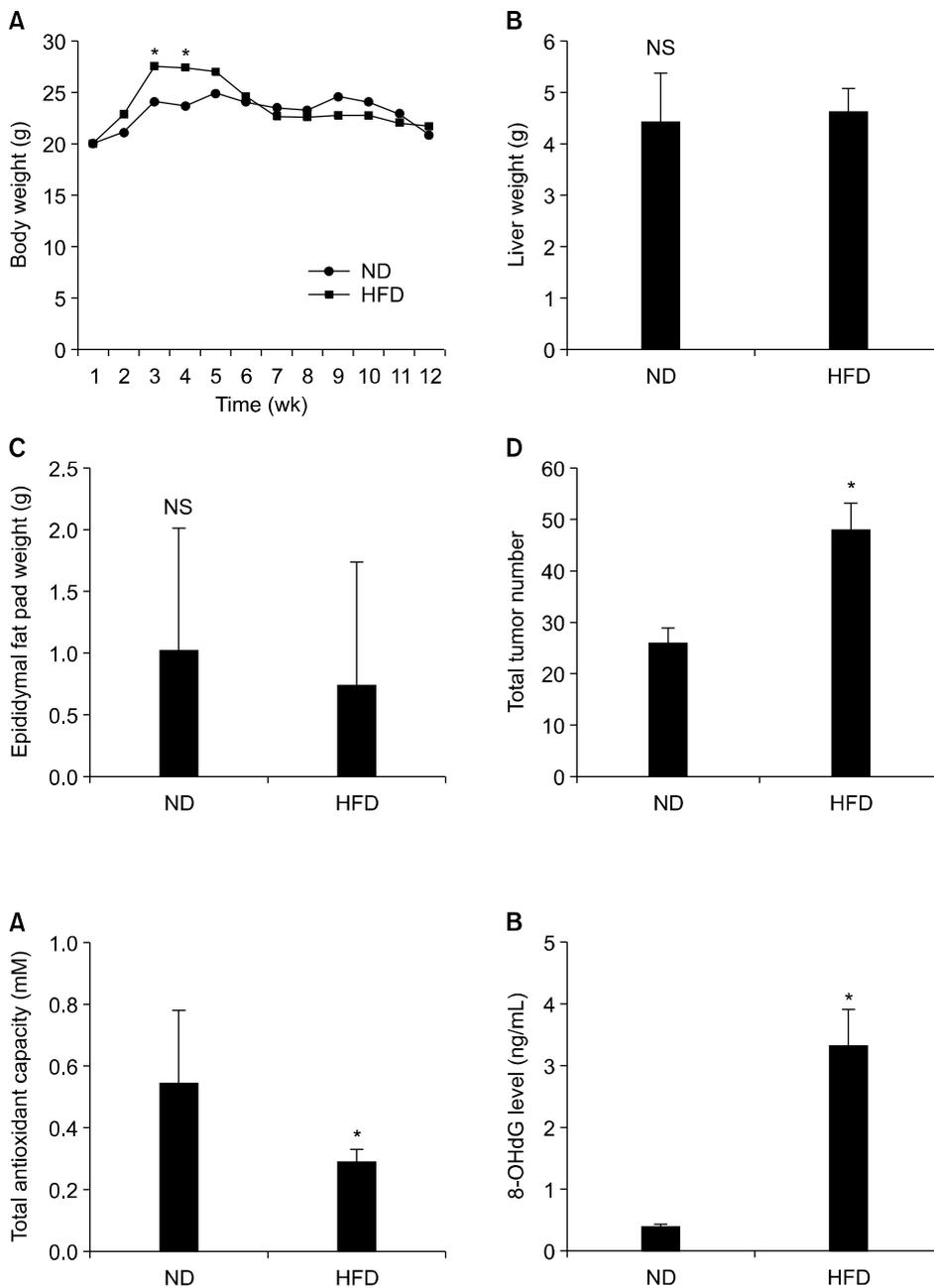


Figure 1. Effects of normal diet (ND) and high-fat diet (HFD) on body weight, tissue weight, epididymal fat pad weight, and total tumor number in *APC^{min/+}* mice. *APC^{min/+}* mice were fed either ND or HFD for 12 weeks. (A) Body weight, (B) liver weight, (C) epididymal fat pad weight, and (D) total tumor number was measured. Values are expressed as mean \pm SD (n = 10). *Significant ($P < 0.05$).

Figure 2. Effects of normal diet (ND) and high-fat diet (HFD) on blood total antioxidant capacity and colonic 8-hydroxy-2'-deoxyguanosine (8-OHdG) level in *APC^{min/+}* mice. *APC^{min/+}* mice were fed either ND or HFD for 12 weeks. (A) Blood total antioxidant capacity and (B) colonic 8-OHdG level were evaluated. Values are expressed as mean \pm SD (n = 10). *Significant ($P < 0.05$).

free radical-induced oxidative lesions in DNA which has been widely used as a biomarker for systemic oxidative stress possibly associated with carcinogenesis. Study results indicated that *APC^{Min/+}* HFD group possessed a half TAC compared with *APC^{Min/+}* ND group, indicating that oxidative stress is significantly increased in HFD group ($P < 0.05$, Fig. 2A). In addition, 8-OHdG level in colon tissue was dramatically increased in HFD fed *APC^{Min/+}* mice compared with tissue 8-OHdG level in ND fed *APC^{Min/+}* mice ($P < 0.05$; Fig. 2B). These data indicated that a substantial colonic DNA damage is induced by HFD-associated oxidative stress.

3. Effect of high-fat diet on intestinal integrity

Study results showed approximately a four-fold increase in serum FITC-dextran in HFD fed group, demonstrating accelerated transepithelial passage of FITC-dextran in this group ($P < 0.05$; Fig. 3A). The TJ complex is a cluster of proteins that forms a

physiologically active barrier at the level of the intestinal epithelial cell, changing its permeability based on the cellular environment. Key TJ proteins include ZO-1, occludin, and claudins. Since the altered paracellular permeability involves a disrupted intestinal barrier, TJ protein expressions were measured by Western blot analysis. Results showed that protein expressions of ZO-1, occludin, and claudin-1 were significantly decreased in HFD fed *APC^{Min/+}* mice compared with those of ND *APC^{Min/+}* mice ($P < 0.05$; Fig. 3B~3D). These data indicate that increased intestinal permeability in HFD *APC^{Min/+}* mice group might be due to the HFD-induced disruption of TJ proteins.

4. Effect of high-fat diet on blood CD14 and colonic toll-like receptor 4 mRNA level

LPS is a component of the cell wall of gram-negative bacteria and is known as endotoxin. In an effort to indirectly determine endotoxemia, CD14, the LPS receptor, was measured in serum. As

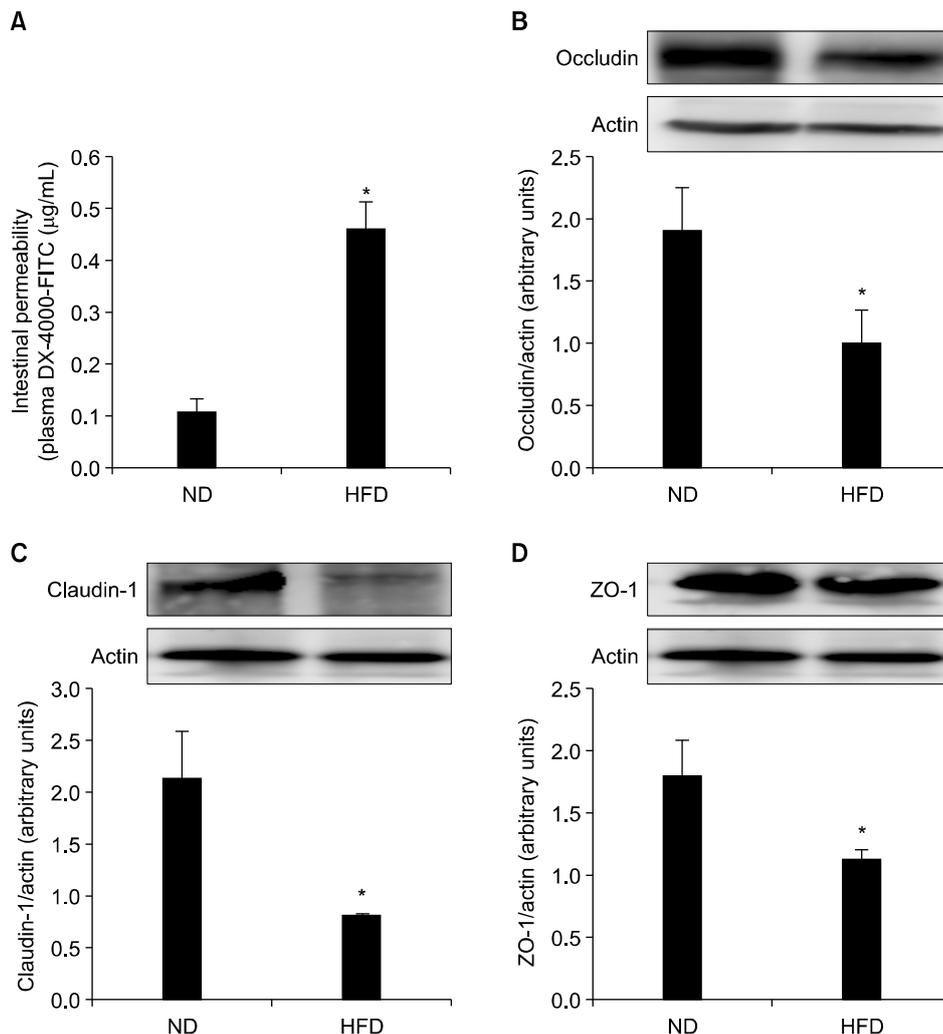


Figure 3. Effects of normal diet (ND) and high-fat diet (HFD) on intestinal permeability and colonic tight junction (TJ) protein levels in *APC^{Min/+}* mice. *APC^{Min/+}* mice were fed either ND or HFD for 12 weeks. (A) Intestinal permeability was assessed by measuring blood fluorescein isothiocyanate (FITC)-dextran level after gavage FITC-dextran (600 mg/kg body weight). Colonic expressions of tight junction proteins, (B) occludin, (C) claudin-1, and (D) zonula occludens-1 (ZO-1), were evaluated by Western blotting. Graph shows ratios of the intensities of TJ protein bands normalized with that of β -actin band. Values are expressed as mean \pm SD (n = 10). *Significant ($P < 0.05$).

LPS is a ligand of TLR4 in the epithelium, the mRNA expression of TLR4 was determined in colon tissue. Figure 4 showed that *APC^{Min/+}* mice fed HFD had significantly higher concentration of serum CD14 than mice fed ND ($P < 0.05$; Fig. 4A). A two-fold increase mRNA expression of TLR4 in colon tissue was also observed in *APC^{Min/+}* mice fed HFD ($P < 0.05$; Fig. 4B).

5. Effect of high-fat diet on blood interleukin-6 level and myeloperoxidase activity

Plasma IL-6 level and MPO level are shown in Figure 5. Plasma IL-6 concentration was significantly increased in *APC^{Min/+}* mice fed HFD ($P < 0.05$; Fig. 5A). MPO level, an index of granulocytic infiltration, was also significantly increased in the HFD fed *APC^{Min/+}* mice ($P < 0.05$; Fig. 5B). These results indicate that HFD significantly accelerates inflammatory response in *APC^{Min/+}* mice which is possibly linked to tumorigenesis.

DISCUSSION

The aim of this study was to provide mechanistic insights for HFD mediated intestinal tumorigenesis. Three weeks after the experimental diet began, animals on HFD started to gain significantly more weight. However, differences in body weight between groups became insignificant starting at week 6. Mice in both experimental groups may have developed polyps as we observed bloody stools. Tumor-induced weight loss is a common feature of cancer and is caused by wasting of muscle and adipose tissue. Depletion of body fat is caused by either the inhibition of the lipoprotein lipase or the stimulation of triglyceride hydrolysis.²² A decreased rate of protein synthesis and enhanced protein degradation also contributed to protein depletion. These may result in the loss of body weight as they start to bear polyps. Concurrently, HFD itself can remain to directly affect the integrity of intestinal epithelium by generating ROS followed by creating inflammatory environment. Previous studies have also indicated that the increased production of secondary bile acids

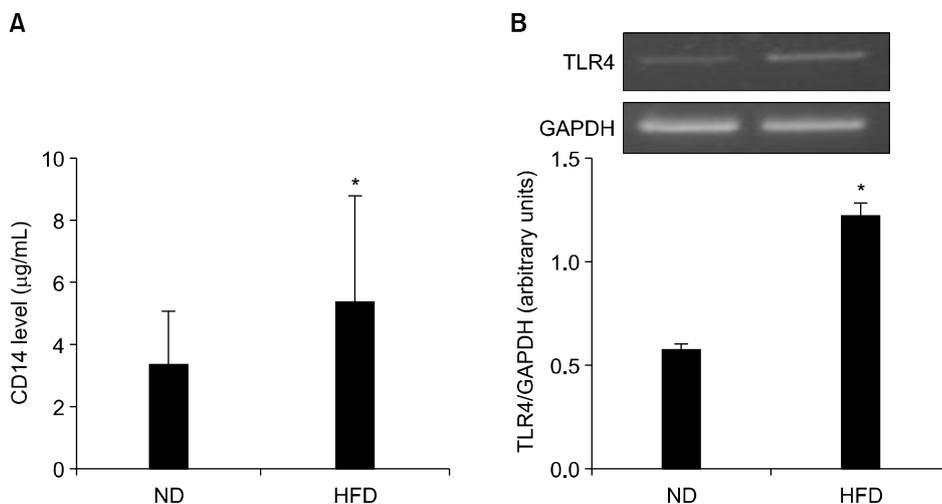


Figure 4. Effects of normal diet (ND) and high-fat diet (HFD) on blood CD14 level and colonic toll-like receptor 4 (TLR4) mRNA expression levels in *APC^{Min/+}* mice. *APC^{Min/+}* mice were fed either ND or HFD for 12 weeks. (A) CD14 level in blood was measured by commercially available ELISA kit. (B) Colonic TLR4 gene expression level was evaluated by RT-PCR. Graph shows ratios of TLR4 band intensity normalized with β -actin band. Values are expressed as mean \pm SD ($n = 10$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *Significant ($P < 0.05$).

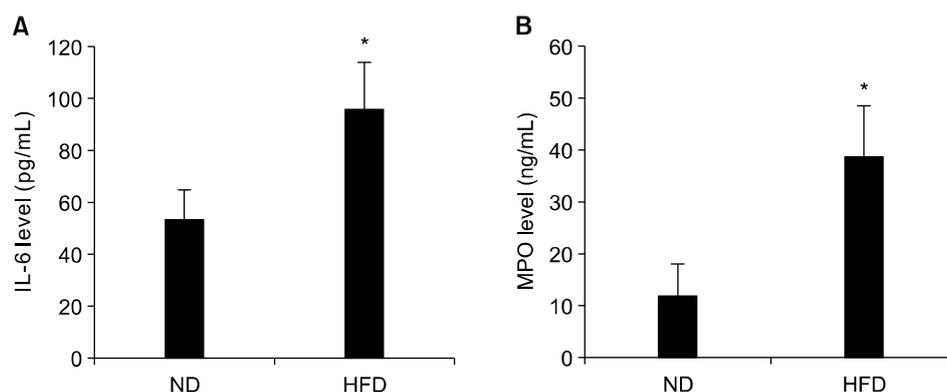


Figure 5. Effects of normal diet (ND) and high-fat diet (HFD) on blood interleukin (IL)-6 level and myeloperoxidase (MPO) level in *APC^{Min/+}* mice. *APC^{Min/+}* mice were fed either ND or HFD for 12 weeks. Blood (A) IL-6 and (B) MPO levels were measured by commercially available ELISA kit. Values are expressed as mean \pm SD ($n = 10$). *Significant ($P < 0.05$).

accelerates the colon tumor formation.²³

It is reported that increased fat storage is linked with increased generation of ROS.²⁴ ROS can interact with DNA to produce damage including single and double-stranded DNA breaks and nucleotide modifications. The level of 8-OHdG, the oxidized form of the nucleoside 2'-deoxyguanosine present in DNA, is one of the most reliable and abundant markers for free radical-induced oxidative lesions.²⁵ Study results indicated that total antioxidant capacity was lower in the HFD group than those of the ND group, while 8-OHdG level was higher in the HFD group. These results show that mice fed HFD were under a heavier oxidative stress and antioxidant defenses might be weakened than mice fed ND.

ROS may also be one of the contributor in gut dysfunction.²⁶ Increased ROS can rapidly stimulate compartmental redistribution of TJs such as occludin and ZO-1 in Caco-2 cells.²⁷ The disruption of TJs plays an important role in the pathogenesis of a number of gastrointestinal diseases including inflammatory bowel disease, celiac disease, allergy, and cancer.^{27,28} In this study, HFD remarkably increased passage of FITC-dextran and significantly decreased expression levels of TJ proteins including occludin, ZO-1, and claudin-1. A previous study has indicated that HFD causes intestinal eosinophil depletion which could result in defective barrier function.²⁹ Not only to promote intestinal tumorigenesis, but severe gut barrier dysfunction enhances progression of colon cancer cachexia in the *Apc^{Min/+}* mice model through increased endotoxemia and higher circulating concentration of IL-6.³⁰ Increased intestinal permeability permits diffusion of bacterial endotoxins such as LPS into the blood stream which is associated with systemic inflammation and development of several diseases.^{20,31} Previous reports showed that inflammation markers were decreased in ob/ob mice lacking LPS receptor CD14 and identified HFD-induced LPS is a triggering factor in metabolic endotoxemia.^{20,32} In a case-control study, blood endotoxin levels were shown to be correlated with colon polyps.³³ We clearly found that HFD significantly increased blood CD14 level and colonic TLR4 mRNA expression level. Among several TLRs, TLR4 is stimulated by binding bacterial LPS. The TLR4/NF- κ B signaling pathway is known to stimulate the secretion of inflammatory cytokines including IL-6 and IL-8, and TLR4-dependent inflammatory response contributed to the colorectal cancer (CRC) development.³⁴ A previous study also showed that the up-regulated TLR4 and its polymorphism are associated with progression of colon cancer.³⁵ TLR4 was also suggested as an important CRC specific cell-surface marker in colorectal cancer patients.³⁶ As LPS treatment increases paracellular permeability in intestinal epithelial cells,³⁷ it can be

postulated that HFD facilitates LPS production and LPS-induced signaling causes endotoxemia, resulting in increased intestinal permeability possibly accelerating CRC progression.

In this study, serum IL-6 concentration and MPO level were used as inflammatory markers. Among several inflammatory cytokines, IL-6 is known as a predictive marker for CRC progression.^{38,40} MPO is a specific marker of neutrophils infiltration, which can be considered as an inflammatory damage index.^{41,42} As expected, both serum IL-6 and MPO level were elevated in the HFD group compared to the ND group in accordance with increased permeability markers.

In summary, HFD induced oxidative stress and endotoxemia, leading to disruption of intestinal barrier in *APC^{Min/+}* mice. In addition, HFD increased both oxidative stress and LPS-related markers. These results indicate that increased intestinal permeability allows entrance of bacterial pathogens and may cause chronic inflammation which accelerates the formation of intestinal polyps in *APC^{Min/+}* mice. This study is one of a few studies suggesting that HFD influences intestinal tumorigenesis by increasing intestinal permeability via oxidative stress and endotoxemia.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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