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Article

Dietary Mixed Cereal Grains Ameliorate the Azoxymethane and Dextran Sodium Sulfate-Induced Colonic Carcinogenesis in C57BL/6J Mice

Jia-Le Song [1,2,3,*,†], Chengqiang Wang [1,†], Jung-Sook Lee [3,5], Byung-Jin Jeong [6], Jong-Sung Jeong [6], Tae-Gon Huh [6] and Kun-Young Park [3,4,*]

[1] Department of Nutrition and Food Hygiene, School of Public Health, Guilin Medical University, Guilin, Guangxi 541004, People's Republic of China; 13730619@qq.com(C. W.)
[2] Department of Surgery, School of Medicine, University of Maryland, Baltimore 21201, Maryland, USA
[3] Department of Food Science and Nutrition, Pusan National University, Busan 609-735, South Korea
[4] Department of Food Science and Biotechnology, CHA University, Seongnam-si, Gyeonggi-do, South Korea
[5] Department of Nutrition, Pusan National University Hospital, Busan 602-739 South Korea; LeeJSPNU@hotmail.com(J.-S. L.)
[6] Doobo Food Co. Ltd, Seoul 137-893, South Korea; JeongBJDOB@hotmail.com(B.-J.J.); JeongJSDOB@hotmail.com(J.-S. J.); HuhTGDOB@hotmail.com(T.-G. H.)
* Correspondence: songjiale@glmc.edu.cn; Tel:+86-181-7836-0021(J.-L. S.) and kunypark@cha.ac.kr; Tel.: +82-31-881-7159 (K.-Y. P.)
† These authors contributed equally to this work.

Abstract: The chemopreventive effects of various mixed cereal grain (MCG) samples on azoxymethane (AOM, 10 mg/kg) and dextran sulfate sodium (DSS, 2% w/v)-induced colorectal cancer (CRC) in C57BL/6J mice were studied. The main MCG preparation consisted of fermented brown rice (FBR), glutinous brown rice, glutinous *Sorghum bicolor*, glutinous *Panicum miliaceum*, *Coix lacryma-jobi* and black soybean at an appropriate mixing ratio. Other MCG preparations contained rice coated with 5% *Phellinus linteus* and 5% *Curcuma longa* (MGR-PC), or 10% *Phellinus linteus* (MCG-P), or 10% *Curcuma longa* (MCG-C). Consumption of dietary MCG-PC by CRC mice significantly increased colon length, decreased the ratio of colon weight to length, and reduced the number of colon tumors. Similar effects, although to a lower extent, were observed in CRC mice fed with MCG-P, followed by those fed with MCG-C, MCG, FBR or white rice (WR). MCG-PC significantly suppressed colonic neoplasia, and decreased the levels of various cytokines (tumor necrosis factor: Tnf, interleukin 1 beta: Il1b, interleukin 6: Il6, and interferon gamma: Ifng) in serum and colon tissue of the CRC mice. In addition, MCG-PC increased the mRNA expressions of tumor protein p53(Tp53) and cyclin-dependent kinase inhibitor 1A(Cdkn1a), activated pro-apoptotic caspase 3(Casp3), and reduced expression of both mRNA and protein of inducible inducible nitric oxide synthase 2 (Nos2), prostaglandin-endoperoxide synthase 2 (Ptgs2), and cyclin D1(Ccnd1) in colon tissue. These findings suggest that than compared with other cereal grain preparations, MCG-PC had a greater activity against AOM/DSS-induced CRC by reducing intestinal inflammation, and modulating the expression of certain carcinogenesis related factors (Nos2, Ptgs2, Tp53, Cdkn1a, Ccnd1 and Casp3) in colon tissue of CRC mice.

Keywords: mixed cereal grains; pro-inflammatory cytokines; inflammation; colon carcinogenesis

1. Introduction

Colorectal cancer (CRC) is among most commonly diagnosed cancer worldwide, and over 1.8 million new CRC cases and 881,000 deaths are estimated to have occurred in 2018 [1]. In Korea, the annual CRC incidence rates increase from 26.2 to 40.4% in men, and 16.4 to 22.4% in women between 1999 and 2016 [2]. Additionally, CRC became the third most common cancer in females and for the first time ranked higher than stomach cancer, which had been the long-standing third most common

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cancer in Korea [3]. The chronic inflammatory bowel diseases (IBDs), such as ulcerative colitis and Crohn's disease, have been known as the important risk factors associated with the pathogenesis of CRC [4]. Overexpressed inflammation-related enzymes, including inducible nitric oxide synthase 2 (Nos2) and prostaglandin-endoperoxide synthase 2 (Ptgs2), promote the development of human CRC. Additionally, these enzymes have also been found to be closely associated with the mechanism whereby chemical carcinogens, such as azoxymethane (AOM) and dextran sulfate sodium (DSS) induce colon carcinogenesis in rodent models [5]. They also induced the production of pro-inflammatory cytokines, including tumor necrosis factor (TNF), interferon (IFNG) interleukin (IL1B), IL6 and IL12, which play a critical role in the initiation and perpetuation of CRC [6]. Other important events, such as overexpression of cell-cycle related cyclin D1(Ccnd1) and dysfunction of wild-type p53, have been found to lead to tumor cell proliferation during CRC carcinogenesis [7,8].

Nutritional intervention is an important tool in disease prevention. For example, approximately 30-40% of cancers can be prevented by dietary intervention [9]. Some epidemiological evidence suggests that whole cereal grain consumption significantly reduces the risk of colorectal cancer [10,11]. Cooked white rice (WR) is a major staple food, which accounts for more than 60% of caloric intake in the traditional Korean diet. However, some studies have found that substituting WR with certain health-promoting cereal grains enhanced the cancer-protective benefits of the Korean diet [12,13]. Brown rice is gradually being recognized as a nutritious food that has many health benefits compared with polished WR [14]. Brown rice can reduce the growth of human colon cancer SW 480 cells, cervical cancer HeLa cells, leukemia Molt4 cells and mouse leukemia L1210 cells in vitro [15,16] and significantly suppressed many serious cancers in experimental rodent models [14,17]. In addition, Tantamango *et al*. has reported that consumption of brown rice at least once a week reduced the risk of colorectal polyp formation by 40% [18]. In particular, brown rice fermented with *Aspergillus oryzae* was able to attenuate the inflammation-related cancer development in rodent models [19,20], suppress the multiplicity of colon tumors in DSS-treated Apc^{min} mice [21], and reduced the multiplicity of colon adenocarcinomas by inhibiting the aberrant crypt foci formation in Fisher 344 rats [22]. In our previous studies, we have reported that brown rice, *Sorghum bicolor*, *Coix lacryma-jobi*, *Panicum miliaceum* and black soybean showed great antimutagenic and anticancer activity [12,23]. In addition, we also reported that brown rice enriched with mixed cereal grains showed an excellent free radical scavenging activity and effectively protected against H_2O_2-induced oxidative damage in LLC-PK1 cells [24]. Also, the modulation of ingredient composition in MCGs, such as by adding *Phellinus linteus* and *Curcuma longa* extracts, was found to enhance their activity against AOM- and DSS-induced colon cancer in mice [25]. Accordingly, in this study, we used a murine model of AOM- and DSS-induced colitis associated colon cancer, which exhibited the same symptoms as those of human CRC [26] and investigated the CRC preventive effects of certain MCGs as well as their underlying mechanism of action.

2. Materials and Methods

2.1. Cereal Samples Preparation and Chemical Reagents

FBR, WR, MCGs were obtained from Doobo Food Co. Ltd. (Seoul, South Korea). Detailed composition of these MCGs is listed in Table 1. For preparing the cooked MCGs, samples were mixed with water and then cooked in a rice cooker (Liho-cuchen Co. Ltd., Seoul, South Korea). Subsequently the cooked MCGs were cooled at room temperature, freeze-dried and powdered. The composition of the AIN-93G diet mixed with 40% of various cooked cereal samples is shown in Table 2. Trizol reagent, OligodT18 primer, murine maloney leukemia virus (MMLV) reverse transcriptase, RNase inhibitor, ethidium bromide (EtBr), and agarose were purchased from Invitrogen (Carlsbad, CA, USA). DSS (molecular weight: 36,000-50,000) was obtained from MP Biomedical (Solon, OH, USA). AOM was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All reagents were of analytical grade.

Table 1. Composition of mixed cereal grains

Ingredient (g/100g)	MCG*-PC	MCG-P	MCG-C	MCG

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Fermented brown rice	40	40	40	50
Glutinous brown rice	20	20	20	20
Glutinous *Sorghum bicolor*	10	10	10	10
Glutinous *Panicum miliaceum*	10	10	10	10
Black soybean	8	8	8	8
Coix lacryma-jobi	2	2	2	2
Rice coated with *Phellinus linteus*	5	10	0	0
Rice coated with *Curcuma longa*	5	0	10	0

* MCGs: Mixed cereal grains

Table 2. Composition of experimental diets

Ingredient (g/1,000g diet)	**Norm***	**WR**	**FBR**	**MCGs**
Corn starch	397.5	54.8	54.8	54.8
Casein	200.0	165.1	165.1	165.1
Dextrinized corn starch	132.0	132.0	132.0	132.0
Sucrose	100.0	100.0	100.0	100.0
Soybean oil	70.0	60.5	60.5	60.5
α-cellulose	50.0	42.1	42.1	42.1
Mineral mix	35.0	30.0	30.0	30.0
Vitamin mix	10.0	10.0	10.0	10.0
L-Cystine	3.0	3.0	3.0	3.0
Choline Bitartrate	2.5	2.5	2.5	2.5
tert-Butylhydroquinone (TBHQ)	0.014	0.014	0.014	0.014
White rice	0	400	0	0
Fermented brown rice (FBR)	0	0	400	0
Mixed cereal grains (MCG)	0	0	0	400

*Norm: AIN-93G diet; WR: AIN-93G diet containing 40% white rice; FBR: AIN-93G diet containing 40% fermented brown rice; MCG-PC, MCG-P, and MCG-C: AIN-93G diet containing 40% of functional mix-cereals as described on Table1.

2.2. Animal Studies and Grouping

Male C57BL/6J mice (6-week old, 16-18 g) were purchased from Samtako Bio Korea (Kyung-ki-do, South Korea). The mice were housed under a standard 12-h light/dark cycle at room temperature, and had access to food and water ad libitum. The animals were randomly divided into eight groups of ten mice each: group 1, mice treated with AIN-93G diet; group 2, AOM and DSS-treated mice; and groups 3~8, AOM and DSS-treated animals fed with AIN-93G diet containing 40% of various WR, FBR and MCG samples. Experimental diets were administered from the 3rd week until mice were sacrificed. To induce the development of colitis-associated colon cancer, the mice were given a single intraperitoneal (i.p.) injection of AOM (10 mg/kg body weight) on the first day of the experiment. Two weeks later, the animals were given DSS (2%, w/v) in the drinking water for the 3rd and 6th week of the experiment. At the end of the experiment (14th week), the mice were euthanized using CO_2 followed by cervical dislocation and tissues of interest were collected. Colon length and weight, and the number of tumors formed were determined. The animal protocol used in this study was reviewed and approved by the Pusan National University Institutional Animal Care and Use Committee (PNU-IACUC; approval number PNU-2011-000408).

2.3. Histological Observations

Four samples of the distal colon from each animal were subjected to histological examination. The colon tissues were fixed in 10% (v/v) neutral buffered formalin, dehydrated in ethanol, and embedded in paraffin. Sections (4-μm thick) were then prepared and stained with hematoxylin and eosin (H & E).

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Optical microscopy images were acquired using a Zeiss Axioskop 2 Plus microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) equipped with an AxioCam MRc5 CCD camera (Carl Zeiss GmbH).

2.4. Measurement of Serum Pro-inflammatory Cytokine Levels

For the serum pro-inflammatory cytokine assay, blood collected from the inferior vena cava was transferred to a tube and centrifuged (3,000 × g for 10 min at 4°C). Serum levels of Tnf, Il1b, Il6 and Ifng were measured with a commercial ELISA MAX kit (Biolegend, San Diego, CA, USA) according to the manufacturer's protocol.

2.5. Reverse Transcription Polymerase Chain Reaction (RT-PCR) Assay

Colonic mRNA expression of *Tnf*, *Il1b*, *Il6*, *Ifng*, *Nos2*, and *Ptsg2*, *Tp53*, *Cdkn1a*, *Casp3* and *Ccnd1* was determined by RT-PCR analysis. Total RNA was isolated from the colonic tissue (100 mg) using the Trizol reagent according to the manufacture's recommendations and centrifuged at 12,000 × g for 15 min at 4°C after the addition of chloroform. Afterwards, isopropanol was added to the supernatant at a 1: 1 ratio and the RNA was pelleted by centrifugation (12,000 × g for 15 min at 4°C). After washing with ethanol, the RNA was solubilized in diethyl pyrocarbonate-treated RNase-free water and quantified by measuring the absorbance at 260 nm using a UV-2401PC spectrophotometer (Shimadzu Corp., Kyoto, Japan). Equal amounts of each RNA (1 μg) sample were reverse transcribed in a master mix containing 1 × reverse transcriptase buffer, 1 mM dNTPs, 500 ng of oligodT_{18} primers, 140 U of MMLV reverse transcriptase, and 40 U of RNase inhibitor for 45 min at 42°C. PCR was then performed in an automatic thermocycler (BIONEER, Daejeon, South Korea) for 25~30 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 40 s) followed by an 8-min extension at 72°C. The PCR products were separated in 2% agarose gels and visualized by EtBr staining. Actin beta (*Actb*) was used for normalization. Gene expression was quantified using the ImageJ software (NIH, Bethesda, MD, USA).

2.6. Protein Extraction and Western Blot Analysis

Colonic tissue samples (100 mg) were first washed with ice-cold phosphate-buffered saline (PBS), homogenized with ice-cold radioimmunoprecipitation assay (RIPA) buffer, and then centrifuged at 12,000 × g for 20 min at 4 °C. Protein concentration of each sample was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). For Western blot analysis, 50 μg of protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA). The blots were incubated with antibodies against Nos2(sc-7271), Ptgs2(sc-19999), Casp3(sc-7272) and Ccnd1(sc-450) obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The blots were washed three times with PBS containing 0.05% v/v Tween 20 (PBS-T) and antibody binding was visualized by enhanced chemiluminescence (GE Healthcare Life Sciences, Little Chalfont, UK). Protein expression was quantified using the ImageJ software (NIH).

2.7. Statistical Analysis

All data are presented as the mean ± standard deviation (SD). Differences between the mean values for individual groups were assessed with a one-way analysis of variance (ANOVA) and Duncan's multiple range tests. Differences were considered significant when $p< 0.05$. The SAS v9.1 statistical software package (SAS Institute Inc., Cary, NC, USA) was used for these analyses.

3. Results

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3.1. Effects of MCGs on Colon Length, Colon Weight/length Ratio and Number of Tumors

As shown in Figure 1a, the total length of the colon of the AOM/DSS-treated mice (5.3 ± 0.5 cm) was significantly shorter than that of the normal mice (7.9 ± 0.5 cm, $p< 0.05$). Treatment with MCG-PC (7.3 ± 0.7 cm) and MCG-P (7.2 ± 0.7 cm) more effectively counteracted the AOM/DSS-induced shortening of the colon than treatment with MCG-C (7.0 ± 1.1 cm), MCG (6.7 ± 0.7 cm), FBR (6.2 ± 0.5 cm) and WR (5.9 ± 0.5 cm).

The ratio of colonic weight to colon length was significantly increased in the AOM/DSS-treated mice, indicating that AOM/DSS induced intestinal wall thickening, inflammation and neoplasia development during the CRC carcinogenesis. The data presented in Figure 1b reveal that treatment with AOM/DSS significantly ($p<0.05$) increased the colon weight/length ratio (to 39.0 ± 3.0 mg/cm) compared with the normal mice (17.5 ± 5.1 mg/cm). Among the different cereal treatments evaluated, MCG-PC(21.3 ± 5.0 mg/cm) and MCG-P(22.8 ± 3.0 mg/cm) in particular showed the best counteracting activity to reduce the AOM/DSS-induced increase of the colon weight to length ratio compared with other cereal treatments (MCG-C: 25.8 ± 5.1, MCG: 26.3 ± 4.9, FBR: 29.0 ± 4.7 and WR: 36.8 ± 3.9 mg/cm). In addition, all the cereal treatments were well tolerated by the mice, and no obvious systemic toxicity was observed during the entire period of treatment (data not shown).

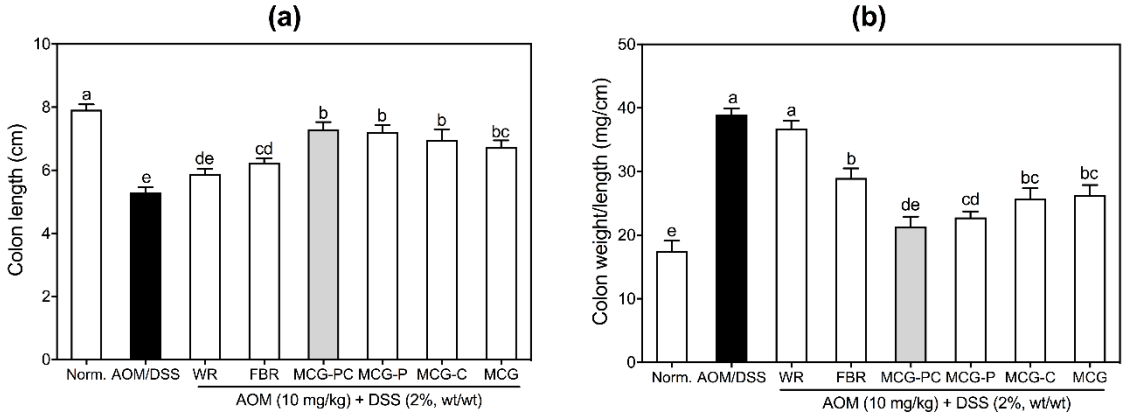

Figure 1. Mixed cereal grains attenuate clinical signs of AOM and DSS-induced colonic carcinogenesis in mice. (a) colon length and (b) colon weight / length ratio. Data are expressed as the mean ± SD. [a–e] Mean values with the different letters are significantly different ($p < 0.05$) according to Duncan's multiple range test.

3.2. Effects of MCGs on Tumor Numbers and Histological Observations in Colon

As shown in Figure 2, the AOM/DSS treatment induced colon carcinogenesis in mice, and produced an average of 17.8 tumors per mouse. Following treatment with these cereal preparations, MCG-PC (10.4 ± 1.8) and MCG-P(10.3 ± 2.5) in particular showed a similar significantly higher ($p<0.05$) antitumor activity against the AOM/DSS-induced tumor formation than MCG-C (11.7 ± 2.1), MCG (12.9 ± 1.7), FBR (13.5 ± 3.1) and WR (15.3 ± 2.0). The results of the histological assay revealed that colon tissue from the mice treated only with AOM/DSS showed typical neoplasia (including the high-grade levels of dysplasia, which were mostly adenoma and adenocarcinoma), loss of crypt and goblet cell depletion. Treatment with MCGs, particularly with MCG-PC and MCG-P reduced the AOM/DSS-induced neoplasia and relieved the infiltration of inflammatory cell into the colon mucosa compared with treatment with other cereal samples (Figure 2).

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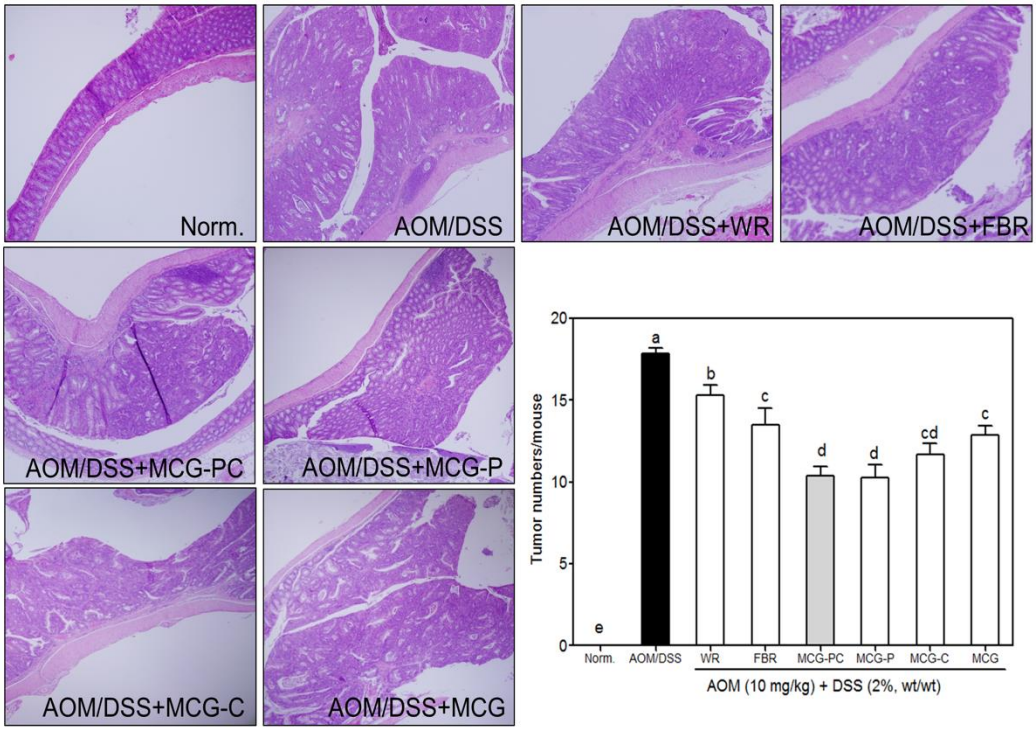

192

193 **Figure 2.** Histological observations and number of tumors of AOM/DSS-induced colitis-associated
194 colon carcinogenesis treated with different mixed cereal grains (MCGs). Colon tissues were obtained
195 at the end of experiment, sectioned, stained with H & E, and observed with an optical microscope
196 (original magnification, 40x). Data are expressed as the mean ± SD. [a~e] Mean values with the different
197 letters are significantly different ($p < 0.05$) according to Duncan's multiple range test.

198 *3.3. Effect of MCGs on Serum Pro-inflammatory Cytokine Levels*

199 The serum levels of the measured CRC-related pro-inflammatory cytokines, namely Tnf, Il1b,
200 Il6 and Ifng are shown in Figure 3. AOM/DSS significantly increased the serum levels of Tnf (6.9-
201 fold), Il1b (2.6-fold), Il6 (3.2-fold) and Ifng (6.3-fold) compared with that in normal mice. The
202 treatment with WR or FBR had only a slight effect on the reduction of the serum levels of Tnf (8.1 and
203 20.7%), Il1b (5.2 and 12.8%), Il6 (1.2 and 15.5%), and Ifng (8.4 and 15.1%) in AOM/DSS-treated control
204 mice. Following treatment with MCGs, in particular MCG-PC and MCG-P significantly reduced the
205 levels of Tnf (34.8 and 32.0%), Il1b (29.4 and 25.3%), Il6 (39.7 and 35.0%), and Ifng (38.8 and 30.5%) in
206 the serum of AOM/DSS-treated mice compared to those found in mice treated only with AOM/DSS.

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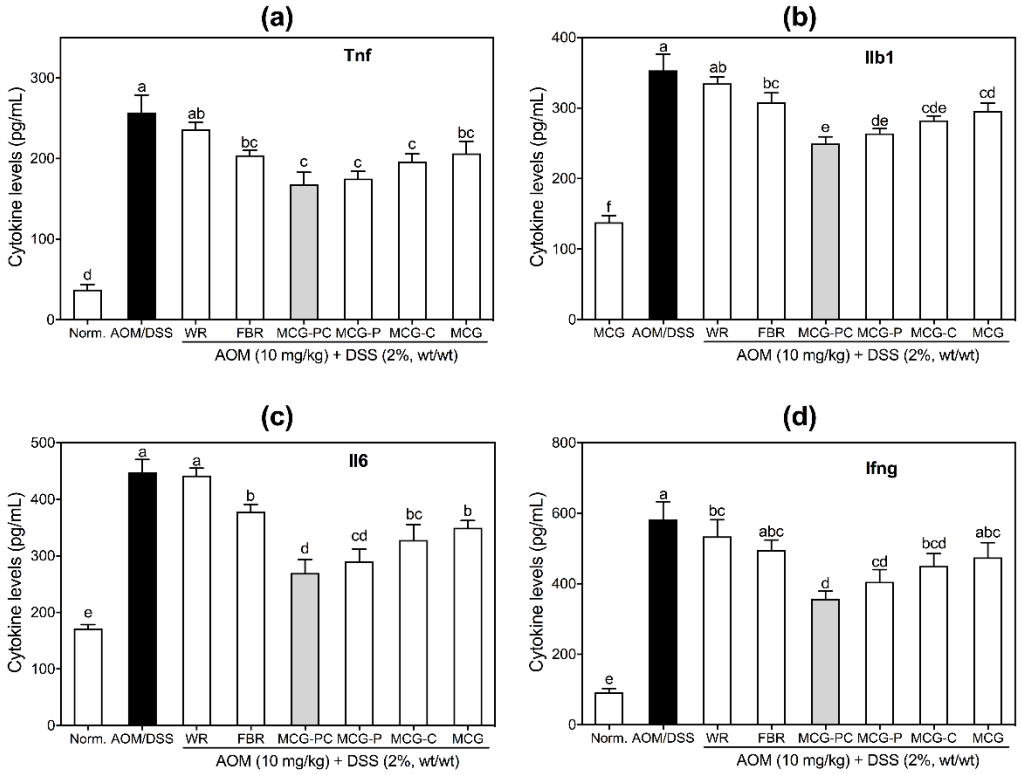

Figure 3. Effects of the mixed cereal grains (MCGs) on serum levels of pro-inflammatory cytokines (a:Tnf, b:Ilb1, c:Il6 and Ifng) in mice treated with AOM (10 mg/kg) and 2% DSS. Data are expressed as the mean ± SD. [a–f]Mean values with the different superscript letters are significantly different (p< 0.05) according to Duncan's multiple range test.

3.4. Effect of MCGs on mRNA Levels of Pro-inflammatory Cytokine in Colonic Tissue

To further investigate the effects of MCGs on AOM/DSS-treated C57BL/6J mice, colonic mRNA expression of Tnf, Il1b, Il6 and Ifng mRNA was determined by RT-PCR analysis. As shown in Figure 4, colonic carcinogenesis induced by AOM/DSS resulted in elevated expressions of all the pro-inflammatory cytokines measured. All the cereal preparations evaluated were able to reduce the AOM/DSS-induced mRNA expression of all these pro-inflammatory cytokines in the colon tissue of CRC mice. The MCG-PC and MCG-P in particular significantly reduced the colonic mRNA levels of Tnf (57.0 and 48.0%), Il1b (62.9 and 60.4%), Il6 (64.5 and 64.9%) and Ifng (50.2 and 44.8%) compared with mice treated with AOM/DSS only. Our findings also revealed that treatment with FBR had a greater effect on reducing the expression of Il1b (28%), Il6 (33%) and Ifng (23%) than treatment with WR in CRC mice.

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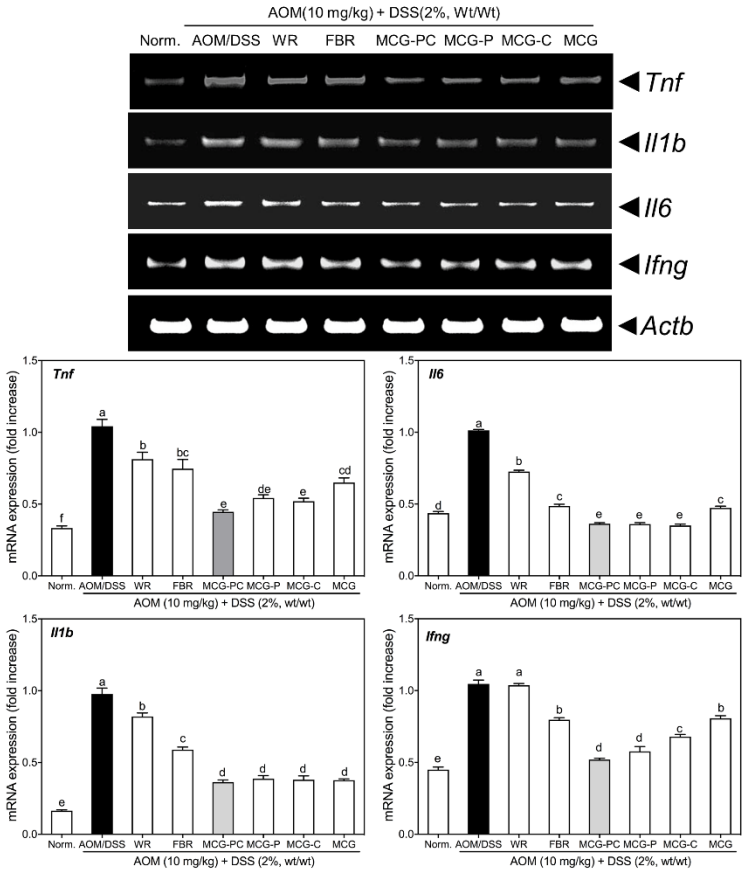

Figure 4. Effects of the mixed cereal grains (MCGs) on mRNA expression of pro-inflammatory cytokines (*Tnf, Il1b, Il6 and Ifng*) in the colonic mucosa of mice treated with AOM (10 mg/kg) and 2% DSS. The PCR products were quantified and normalized to Actb (internal control). Band intensities were measured with a densitometer and expressed as fold change of the control. Fold increase = gene expression/Actb× control value (control fold increase = 1). [a-f]Mean values with different letters over the bars are significantly different (p <0.05) according to Duncan's multiple-range test.

3.5. Effect of MCGs on iNOS and COX-2 Expression in Colon Tissue

AOM/DSS significantly increased the mRNA levels of *Nos2* and *Ptgs2* in the colonic mucosa of mice (Figure 5). Treatment with MCGs, especially MCG-PC significantly reduced the mRNA levels of *Nos2* (72.9%) and *Ptgs2* (86.8%), and also decreased the protein levels Nos2 (30.3%) and Ptgs2 (35.9%) compared to levels found in AOM/DSS-treated control mice. Treatment of CRC mice with other MCGs, including MCG-P, MCG-C and MCG also showed a stronger effect on reducing the expression of Nos2 and Ptgs2 in the colonic mucosa than those of WR and FBR.

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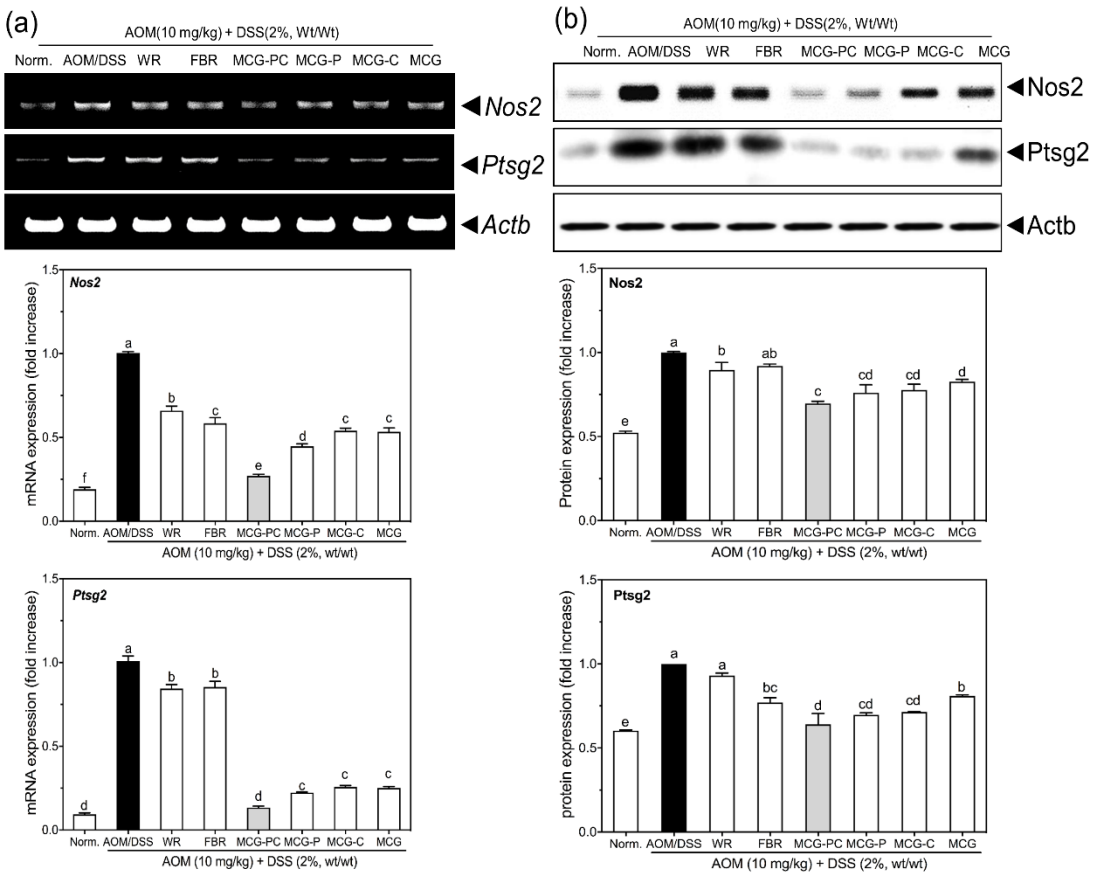

Figure 5. Effects of the mixed cereal grains (MCGs) on (A) mRNA and (B) protein expression of Nos2, and Ptsg2 in the colonic mucosa of mice treated with AOM (10 mg/kg) and 2% DSS. The PCR products and proteins were quantified and normalized to Actb (internal control). Band intensities were measured with a densitometer and expressed as fold change of the control. Fold increase = gene expression/Actb × control value (control fold increase = 1). [a-f]Mean values with different letters over the bars are significantly different ($p < 0.05$) according to Duncan's multiple-range test.

3.6. Effect of MCGs on mRNA Expressions of Cdkn1a and Tp53 in Mouse Colon Tissue

As shown in Figure 6, AOM/DSS treatment significantly decreased the mRNA levels of *Tp53* (67.4%) and *Cdkn1a* (50.7%) compared with those found in the normal mice colonic mucosa. Following treatment with cereal preparations, we noticed that both treatment with MCG-PC and MCG-P effectively increased the *Tp53* (11.6- and 8.4-fold) and *Cdkn1a* (8.8- and 4.6-fold) levels compared to mice treated only with AOM/DSS. However, the FBR and WR treatments had a weaker effect on increasing the mRNA expression of *Tp53* and *Cdkn1a* than that of other MCGs (MCG-C and MCG) in CRC mice.

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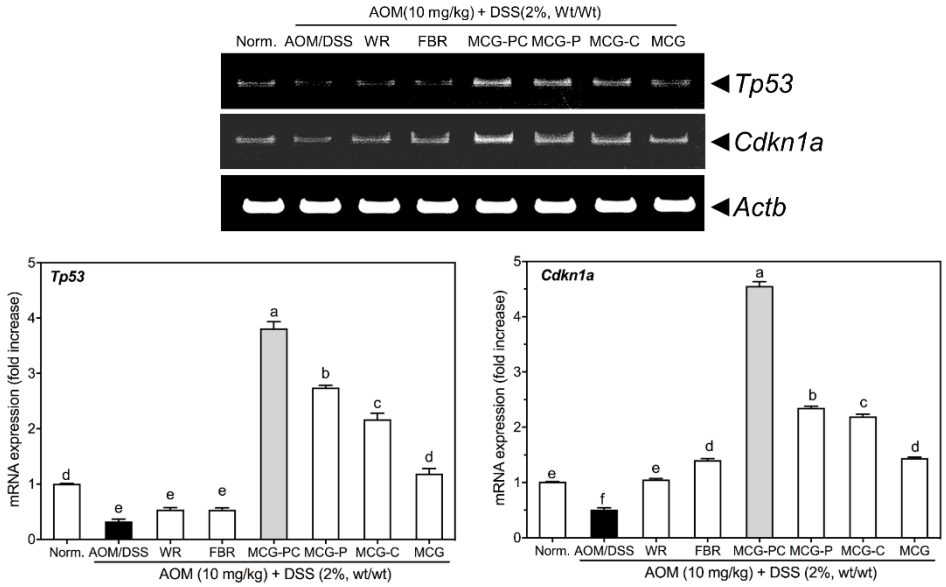

Figure 6. Effects of the mixed cereal grains (MCGs) on the mRNA expression of *Tp53*, and *Cdkn1a* in the colonic mucosa of mice treated with AOM (10 mg/kg) and 2% DSS. The PCR products were quantified and normalized to Actb (internal control). Band intensities were measured with a densitometer and expressed as fold change of the control. Fold increase = gene expression/Actb× control value (control fold increase = 1). [a-e]Mean values with different letters over the bars are significantly different ($p < 0.05$) according to Duncan's multiple-range test.

3.7. Effect of MCGs on Casp3 and Ccnd1 Expression in Colon Tissue

We found that the treatment with MCG-PC increased the colonic expression of Casp3 both at the mRNA (2.8-fold) and protein (2.4-fold) levels compared with the control treatment. Treatment with other MCGs, such as MCG-P and MCG-C also showed a stronger effect on increasing the Casp3 expression than other MCG preparations (FBR and WR) in the colon tissue of the CRC mice (Figure 7). In addition, mRNA and protein levels of Ccnd1 were increased in the colonic mucosa when mice exposed to AOM and DSS treatment (Figure 7). Following treatment with MCGs, in particular MCG-PC, significantly reduced the expression levels of Ccnd1 mRNA (85.7%), and protein (59.7%) compared with the expression levels in CRC mice treated only with AOM/DSS.

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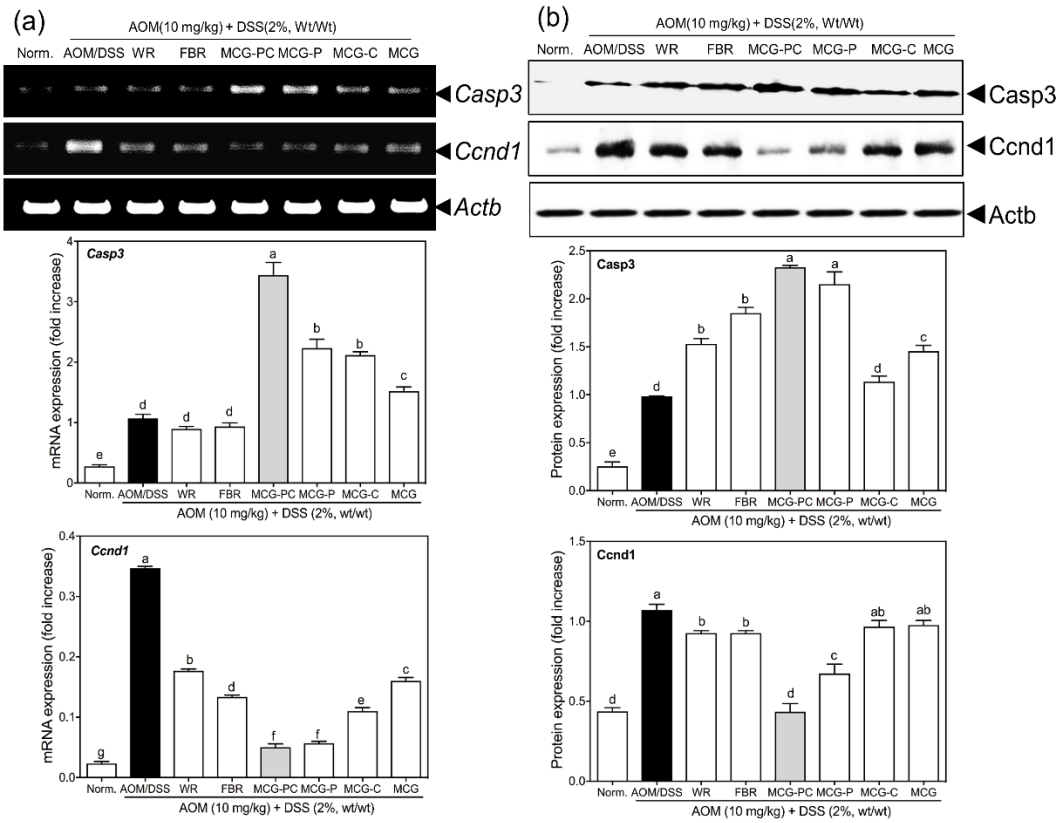

Figure 7. Effects of the mixed cereal grains (MCGs) on (a) mRNA and (b) protein expression of Casp3, and Ccnd1 in the colonic mucosa of mice treated with AOM (10 mg/kg) and 2% DSS. The PCR products and proteins were quantified and normalized to Actb (internal control). Band intensities were measured with a densitometer and expressed as fold change of the control. Fold increase = gene expression/Actb× control value (control fold increase = 1). [a-g]Mean values with different letters over the bars are significantly different ($p < 0.05$) according to Duncan's multiple-range test.

4. Discussion

Many epidemiological studies have suggested that dietary rice and whole cereal consumption is associated with a decreased risk of CRC and also increased the survival rate of patients diagnosed with CRC patients [9,10,27]. The lifestyle and eating habits of South Koreans have been progressively westernized, especially the increased consumption of American and European style diet, while the decreased intake of cereals has led to an increase in CRC in the past 60 years [2,3].

In this study, we observed that treatment of CRC mice with MCGs, especially MCG-PC, significantly reduced the AOM/DSS-induced shortening of the colon length, and the ratio of colon weight to length, to an extent that was comparable to those obtained by treatment with other MCGs (MCG-P, MCG-C and MCG), FBR, and WR. Dietary treatment with 40% of cereal grains also reduced the AOM/DSS-induced tumor formation in C57BL/6J mice. Treatment with MCG-PC significantly decreased the AOM/DSS-induced neoplasia compared to treatment with other MCGs.

The MCG samples used in this study contained high levels of brown rice, including 40-50% of FBR and 20% of glutinous brown rice. High frequency of consumption of brown rice was found to reduce the risk of colorectal polyp formation [18], and to act against colonic carcinogenesis induced by certain colon-specific carcinogens in rodent models[28-30]. FBR, a brown rice product, which is fermented with *Aspergillus oryzae*, also displayed a stronger suppressor activity against colonic carcinogenesis in DSS-treated Apc^{min} mice [21], and Fisher 344 rats [22]. Besides the high levels of brown rice and FBR, MCG-PC, MCG-P and MCG-C also contained higher levels of rice coated with *Phellinus linteus* or rice coated with *Curcuma longa* compared with the common MCG. *Phellinus linteus* has been found to have many pharmacological activities, including antitumor, immunomodulatory,

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anti-inflammatory, anti-allergic, anti-angiogenic and antioxidant effects [31,32], and also effectively reduced the growth of human colon carcinoma cells [33-35]. *Curcuma longa* has also been used as a traditional herbal medicine due to its antiatherosclerotic, antidiabetic, antimutagenic, anticancer, and antioxidant effects [36]. Indeed, many studies have found that *Curcuma longa* has a strong inhibitory activity against colon cell proliferation in vitro, in rodent models of chemical-induced colon carcinogenesis, and also attenuate the symptoms in CRC patients [36,37].

A chronic inflammatory environment in intestinal system results in the impairment of mucosal immunological regulation. In particular the imbalance between pro- and anti-inflammatory cytokines is associated with the development of CRC, and severely shifts toward the pro-inflammatory factors [4]. Suppression of pro-inflammatory cytokines activation can attenuate the inflammatory reaction and reduce colonic carcinogenesis [38]. Here, treatment with FBR and MCGs, in particular MCG-PC, significantly reduced the serum levels of pro-inflammatory cytokines (Tnf, Il1b, Il6 and Ifng), and also reduced the colonic mRNA expression of these cytokines in CRC mice. Blocking of Tnf signaling and suppression of Il6 activation were both able to reduce the colitis-associated colon carcinogenesis in mice [39,40], and improve the survival rate of patients with colon cancer in a clinical study [41]. In addition, dietary FBR also reduced the level of the DSS-induced colonic mRNA expression of Tnf in Apc^{Min} mice [21], and attenuated the DSS-induced colitis in Wistar rats [20].

It is widely recognized that the inflammation-associated enzymes NOS2 and PTGS2 play a major regulatory role in the promotion of human intestinal inflammation, are involved in the development of CRC, and are associated with worse survival among CRC patients [42,43]. Overactivated NOS2 induced the nitric oxide (NO) generation and subsequently increased the PTGS2 activity, thereby increasing prostaglandin E2 production, which increased cell proliferation, promoted inflammatory reaction and inhibited tumor cell apoptosis in CRC carcinogenesis in humans and rodents [44,45]. Treatments with Nos2- and Ptgs2-specific inhibitors were both effective against AOM/DSS-induced colonic carcinogenesis in rodent studies [46,47]. In this study, we found that treatments with FBR and MCGs, especially MCG-PC, effectively reduced the colonic expression levels of Nos2 and Ptgs2 compared to levels found in CRC mice. Dietary FBR was reported to significantly reduce the mRNA expression of Nos2 and Ptgs2 in the colon tissue of DSS-treated Apc^{Min} mice [21]. The effects of some functional compounds found in brown rice have been studied. For example, tricin was found to suppress the AOM/DSS-induced colon carcinogenesis by reducing colonic mRNA expression of Nos2 and Ptgs2 in Crj: CD-1 mice [48], and phytic acid also inhibited colon carcinogenesis by reduction of Ptgs2 and catenin beta 1 (Ctnnb1) in AOM-treated rat [49,50].

Maintenance of the normal function of tumor suppressor protein Tp53 leads to the inhibition of the AOM/DSS-induced colon carcinogenesis in mice [51,52]. The mutated Tp53 prolonged the activation of NF-κB, thereby maintaining the inflammatory reaction and promoting tumor initiation and progression [53]. Treatment with all MCG samples, especially the MCG-PC, increased the colonic mRNA levels of Tp53 in AOM/DSS-treated mice. Activated Tp53 is able to activate the BCL2 binding component 3 (Bbc3, also known as Puma) protein, and enhance the p53-dependent apoptosis to suppress the AOM/DSS-induced colon carcinogenesis in Apc^{Min} mice [54]. CDKN1A is a cyclin-dependent kinase (CDK), which is tightly controlled by TP53, and through inhibition of the CDK/cyclin complex to influence the G1/S cell cycle, and subsequent suppression of the abnormal cell proliferation during the development of CRC [55,56]. Disruption of Cdkn1a leads to a high frequency of colorectal tumorigenesis in AOM-treated mice [57]. In contrast, maintaining the normal function of Cdkn1a reduced the AOM-induced colonic tumorigenesis in mice [58]. Dietary MCGs treatments, in particular MCR-PC, increased the colonic mRNA levels of Cdkn1a compared with those found in CRC mice treated with other MCGs, FBR and WR. Enhancement of the Cdkn1a activation can reduce colon carcinogenesis in AOM and DSS-treated mice [59]. These findings suggested that treatment with MCGs reduced the AOM and DSS-induced colon carcinogenesis in mice, mediated through upregulation of Tp53 and Cdkn1a activation.

Ccnd1 is an important cell-cycle regulated protein, which promotes the G1 to S transition during the cell cycle. Ccnd1 overexpression results in epithelial cells proliferation and has been observed in many human CRC patients [7,8]. Dietary MCG-PC treatment in CRC mice significantly reduced the

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colonic Ccnd1 levels compared with treatment with other MCGs, FBR and WR. *Phellinus linteus* and germinated brown rice extract decreased Ccnd1 production and induced cell cycle arrest at the G0/G1 stage of the cell cycle in human HT-29 colon cancer cells [35]. Reduction of the CDKN1A expression is strongly associated with prolonged survival in male CRC patients [60]. Activated Casp3, which performs the cleavage of the poly (ADP-ribose) polymerase family, member 1 (PARP1) and induces cell apoptosis, is a useful strategy in colon cancer prevention and treatment [61]. In this study, we found that the dietary MCG-PC treatment significantly enhanced the activation of Casp3 in the colon tissue of CRC mice. The high levels of Casp3 activation in the colon tissue is associated with a better survival in human CRC patients [62].

5. Conclusions

In conclusion, the results from the present study indicated that MCGs, in particular MCG-PC dietary administration, significantly reduced the AOM/DSS-induced colonic carcinogenesis in CRC mice. Additionally, MCG-PC administration had a reducing effect on the intestinal inflammatory reaction by suppressing pro-inflammatory cytokines (Tnf, Il1b, Il6 and Ifng) and inflammation-associated enzymes (Nos2 and Ptgs2) in the colon tissue of the mice treated with AOM/DSS. In addition, treatment with MCG-PC increased the mRNA expressions of *Tp53* and *Cdkn1a*, activated the pro-apoptotic Casp3, and also reduced the tumorigenesis-related Ccnd1 to protect mice against development of AOM/DSS-induced colon cancer. The results also suggest that the proportion of functional ingredients in the MCG was associated with the protective effects against chemical-induced carcinogenesis in the colon. These MCGs, especially MCG-PS, could be used as a substitute staple rice diet in human colon cancer prevention.

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