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RESEARCH NOTE

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Antigenotoxic Effect of *Paecilomyces tenuipes* Cultivated on Soybeans in a Rat Model of 1,2-Dimethylhydrazine-induced Colon Carcinogenesis

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Abstract We evaluated the effect of soybean *dongchunghacho* [SD, cultivated *dongchunghacho* fungus (*Paecilomyces tenuipes*) on soybeans] on dimethylhydrazine (DMH)-induced DNA damage and oxidative stress in male F344 rats. The animals were divided into 3 groups and fed a casein-based high-fat, low fiber diet without (DMH group) or with 13%(w/w) of soybean (DMH+S group), or SD (DMH+SD group). One week after beginning the diets, rats were treated weekly with DMH (30 mg/kg, s.c.) for 6 weeks; dietary treatments were continued for the entire experiment and endpoints measured at 9 weeks after the first DMH injection. SD supplementation reduced DMH-induced DNA damage in colon cells and reduced plasma lipid peroxidation. Thus, SD may have therapeutic potential for early-stage colon carcinogenesis.

Keywords: *Paecilomyces tenuipes* cultivated on soybean, 1,2-dimethylhydrazine (DMH), colon carcinogenesis, DNA damage, antioxidant system

Introduction

Colorectal cancer is the third most common malignant neoplasm in the world (1), and is the 4th most common cancer in Korea (2, 3). Although the incidence and mortality of colorectal cancer are lower in Korea than in Western countries, its incidence in Koreans increased from 5.8% among all cancers in 1980 to 11.2% in 2002 (4).

Diet is the greatest contributor to human cancer, including colon cancer, and may be associated with 35-70% of the incidence of the disease (5). For example, high intake of red meat plays an important role in the etiology of colon cancer (6). On the other hand, dietary fiber or phytochemicals may reduce the risk of colon cancer (7, 8). Therefore, much attention has been focused on reducing colon cancer risk through the medicinal properties of natural compounds.

Paecilomyces sp. is a common entomogenous fungus that parasitizes lepidopteran larvae, pupae, and adults, and is common in many mountainous areas in Korea (9). The fruiting body of this fungus is called the snow-flake *dongchunghacho* in Korea because of its appearance (10), and its entomogenous mycelium is widely used in traditional medicines. In spite of the complexities of the life cycle of the fungi imperfecti, artificial cultivation techniques have been developed for these fungi, enabling large-scale production of the fruiting bodies of *Paecilomyces tenuipes* in Korea (11). Its methanol extract has hypoglycemic, immuno-stimulating, and anti-fatigue activities in rat models (12). *P. tenuipes* can induce cellular differentiation and inhibit cell growth in various malignant cell lines (13, 14), and induces apoptosis in a human leukemic cell line (15). Moreover, an extract obtained from the mycelium of *P. tenuipes* inhibited monoamine oxidase, and thus reduces the contribution to oxidant stress made by this reactive species. The extract could potentially control the aging process (16).

We developed newly a technique for cultivating *dongchunghacho* fungus (*P. tenuipes*) on soybean (soybean *dongchunghacho*, SD), which is a useful source of essential amino acids, readily available, and inexpensive (17, 18). In this study, we evaluated the effect of SD on dimethylhydrazine (DMH)-induced DNA damage (by comet assay) and oxidative stress in male F344 rats. DMH, a procarcinogen, undergoes oxidative metabolism in the liver, which results in the production of an active carcinogenic electrophile (the diazonium ion), which is subsequently released into the circulation, eventually culminating in lipid peroxidation in the plasma (19).

Materials and Methods

Reagents Casein, the mineral mixture, and the vitamin mixture were from ICN Pharmaceuticals Inc. (Costa Mesa, CA, USA). Corn starch was obtained from Daesang Co. (Seoul, Korea) and corn oil and lard were from Cheiljedang Co. (Seoul, Korea) and Shinhan Oil Co. (Gosung, Korea), respectively. Tryptic soy broth (TSB) was purchased from Difco Laboratories (Detroit, MI, USA), and cyclohexane was from Merck (Whitehouse Station, NJ, USA). All other chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Fungal strain The entomopathogenic fungus strain used was collected from a mountainous Chocheon village belonging to Miryang (Gyeongnam, Korea), and identified as *Paecilomyces tenuipes* from comparison of ribosomal

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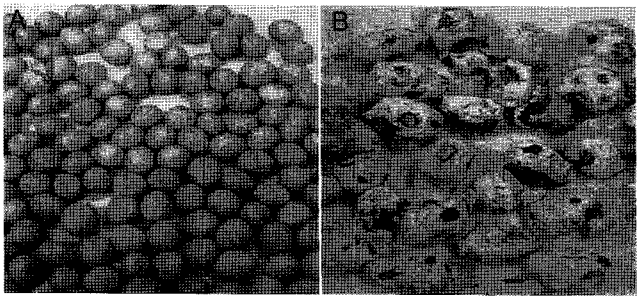

Fig. 1. Image of soybean *dongchunghacho*.
A, before inoculation; B, after inoculation.

DNA internal transcribed spacer regions (data not shown). However, characteristics such as the growth rate of the hyphal body, diameter, length, color of the fruiting body, time from inoculation of the fungus against the silkworm to the harvesting time position of the *dongchunghacho* (insect-mushroom) were very different from the existing *P. tenuipes* (data not shown). Therefore, the fungus was named *P. tenuipes*-Chocheon strain as a variety established from native conditions.

Induction of mycelium and spores on soybeans Soybeans were purchased from a local supermarket in Miryang and used as the substrate for SD production under solid state cultivation. Soybeans were soaked in water for 40 hr. Excessive water was removed with a sieve, and the soaked soybean were measured at 100 g into autoclavable bags and autoclaved for 16 min at 121°C. After cooling, the substrate was inoculated with 1 mL *P. tenuipes*-Chocheon strain (10^8 spores/mL), and the inoculated substrate was cultivated at 24°C for 10 days in a dark incubator, and then 30 days further under a photoperiod of 24 L to induce mycelium and spore growth on the soybeans (Fig. 1). SD was then lyophilized for further use.

Animal and diets Five-week-old male F344 rats (185± 10 g) were purchased from Samtako Inc. (Osan, Gyeonggi, Korea) and were housed individually in hanging wire cages in a room controlled for humidity (55%) and temperature (25°C) were kept under a 12/12 hr light-dark cycle. The animals were cared for in accordance with the 'Guide for Care and Use of Laboratory Animals' (20). The rats were allowed free access to water and were fed a commercially prepared pellet diet for adjustment for the first week. The rats were divided into 3 groups of 10 animals each, and were fed either a high-fat, low-fiber diet (DMH group), or a high-fat, low-fiber diet supplemented with 13%(w/w) of soybean (DMH+S group), or 13% of *P. tenuipes* cultivated on soybean (DMH+SD group) (Table 1). One week after the initiation of the diets, the rats were treated with dimethylhydrazine (DMH, 30 mg/kg, s.c.) for 6 weeks. The dietary treatments were sustained throughout the entire experiment. The general health of the animals was assessed daily and their body weights were recorded every week for the duration of the study. Following the 10-week experimental period, the rats were anesthetized with ethyl ether, with the entire colon collected for aberrant crypt analysis, and blood collected from the abdominal artery and placed in a heparinated sterile tube. Whole blood was freshly prepared for use in the comet assay. Plasma was obtained from the blood samples by centrifugation (700×g for 30 min) and stored at -80°C until further analysis. Erythrocytes were washed 3 times with isoosmotic phosphate buffered saline (PBS), pH 7.4, and resuspended to the original volume. The erythrocyte-suspensions were frozen at -80°C until final analysis.

Table 1. Composition of experimental diets

Ingredients	DMH[1)]	DMH+S[2)]	DMH+SD[2)]
Casein	20	14.8	14.8
Corn starch	52.949	49.749	49.749
Soybean or soybean *dongchunghacho*	-	13	13
Corn oil	10	6.1	6.1
Lard	10	10	10
Cellulose	2	1.3	1.3
Vitamin mixture[3)]	1	1	1
Mineral mixture[4)]	3.5	3.5	3.5
Choline bitartarate	0.25	0.25	0.25
DL-Methionine	0.3	0.3	0.3
Butylated hydroxy toluene	0.001	0.001	0.001
Total	100	100	100

[1)]DMH, high-fat and low-fiber diet+DMH injected group; DMH+S, high-fat and low-fiber diet+soybean+DMH injected group; DMH+SD, high-fat and low-fiber diet+soybean *dongchunghacho*+DMH injected group.
[2)]Soybean contains 28.5 g carbohydrates, 40.1 g protein, 19.7 g fat, 5.5 g fiber, 6.2 g ash per 100 g (31). The experimental diets (DMH+S and DMH+SD) were adjusted to provide the same needs as control diet (DMH).
[3)]AIN 93 vitamin mixture contained (in g/kg of mixture): thiamine HCl 0.6; riboflavin 0.6; pyridoxine HCl 0.7; niacin 3; *d*-calcium pantothenate 1.6; folic acid 0.2; *d*-biotin 0.02; cyanocobalamin (vitamin B_{12}) 0.001; dry vitamin A palmitate (500,000 U/day) 0.8; dry vitamin E acetate (500 U/day) 10; vitamin D_3 trituration (400,000 U/g) 0.25; menadione sodium bisulfite complex 0.15; sucrose, finely powdered, 981.08.
[4)]AIN 93 mineral mixture contained (in g/kg of mixture): calcium phosphate, dibasic 500; sodium chloride 74; potassium citrate, monohydrate 220; potassium sulfate 52; magnesium oxide 24; manganous carbonate (43-48% Mn) 3.5; ferric citrate (16-17% Fe) 6; zinc carbonate (70% ZnO) 1.6; cupric carbonate (53-55% Cu) 0.3; potassium iodate 0.01; sodium selenite 0.01; chromium potassium sulfate 0.55; sucrose, finely powdered 118.03.

Colon cell isolation The colons were isolated, flushed with prewarmed buffer, filled with digestion solution containing protease (50,000 U/L) (Proteinase K, EC 3.4.21.64) and incubated at 37°C for 30 min. Colon cells were shaken free by gentle agitation, centrifuged (at 107×g for 8 min, 4°C), and resuspended in RPMI 1640 medium at 2×10^9 colon cells/L for cytotoxicity and genotoxicity determination.

DNA damage determination by alkaline comet assay The alkaline comet assay was performed according to the protocol established by Singh *et al.* (21), with slight modification. Frosted slides (Fisher Scientific, Pittsburgh, PA, USA) were prepared with a basal layer of 0.5% normal melting agarose (NMA), and colon cells (2×10^5) mixed with 75 μL of 0.7% low melting agarose (LMA) was then added to the slides. The slides were again

covered with coverslips and refrigerated for 10 min. The coverslips were then removed and a top layer of 75 μL of 0.7% LMA was added prior to placing the slides (with coverslips) in the refrigerator for another 10 min. After removal of the coverslips, the slides were immersed in a jar containing cold lysing solution (pH 10), consisting of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylsarcosine; fresh 1% Triton X-100 and 10% DMSO were added to the solution, which was then refrigerated for 1 hr. After lysis, the slides were placed in a horizontal electrophoresis tank (Threeshine Co., Ltd., Daejeon, Korea). The slides were then covered with a fresh alkaline buffer (300 mM NaOH, 10 mM Na_2EDTA, pH 13.0) and incubated at 4°C for 40 min. Electrophoresis of the DNA was executed by applying an electric current of 25 V/300±3 mA for 20 min at 4°C. The slides were washed 3 times with neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4°C, and were then treated with ethanol for another 5 min. All steps following the lysis treatment were performed in the dark to prevent any additional DNA damage. Fifty μL of ethidium bromide (20 μg/mL) was added to each slide, and the slides were then analyzed using a fluorescence microscope (Leica DMLB, Wetzlar, Germany). Images of 100 cells randomly selected from each subject (50 cells from each of 2 replicate slides) were analyzed, and measurements were made by image analysis (Komet 4.0; Kinetic Imaging, Liverpool, UK), to determine the percentage of DNA in the tail, the tail length and the tail moment (TM, calculated as the percentage of DNA in the tail multiplied by the tail length).

Plasma total radical-trapping antioxidant potential (TRAP) The TRAP in plasma was measured using a modification of the photometric method developed by Rice-Evans and Miller (22). The method used to measure antioxidant activity is predicated on the antioxidant-induced inhibition of the absorbance of the radical cation of 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate) ($ABTS^+$). The $ABTS^+$ radical cation is formed by the interaction of $ABTS^+$ (150 μM) with the ferrylmyoglobin radical species, which is generated by the H_2O_2-induced (75 μM) activation of metmyoglobin (2.5 μM). Ten mL of sample/buffer/Trolox-standard were added to tubes containing 400 μL of PBS buffer, 20 μL of metmyoglobin, and 400 μL of ABTS, and the solution was vortexed. The reaction was initiated by the addition of 170 μL of H_2O_2. Following 6 min of incubation, the absorbance was measured at 734 nm using a spectrophotometer (Shimazu, Tokyo, Japan). These values are expressed as the Trolox equivalent antioxidant capacity (TEAC), and are defined as the mM concentration of the Trolox antioxidant capacity of a calibration curve.

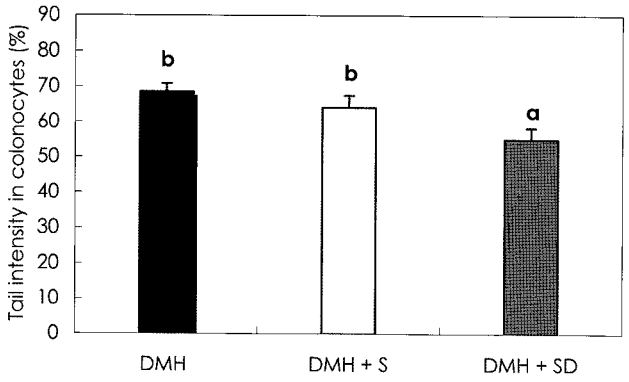

Fig. 2. Effect of soybean *dongchunghacho* on colonocytic DNA damage. DMH, high-fat and low-fiber diet+DMH injected group; DMH+S, high-fat and low-fiber diet+soybean+DMH injected group; DMH+SD, high-fat and low-fiber diet+soybean *dongchunghacho* +DMH injected group. Bars represent mean±SE. Bars with different superscripts are significantly different at the $p<0.05$ level by ANOVA and Duncan's multiple range test.

Baseline conjugated dienes in LDL Baseline low density lipoprotein (LDL)-conjugated diene levels were determined according to the methods outlined by Ahotupa *et al.* (23), with slight modifications. Plasma (100 μL) was added to 700 μL of heparin citrate buffer (0.064 M trisodium citrate, 50,000 IU/L heparin, pH 5.05), and this suspension was incubated for 10 min at room temperature. The insoluble lipoproteins were then sedimented by centrifugation at 1,000×g for 10 min. The pellet was resuspended in 100 μL of 0.1 M Na-phosphate buffer containing 0.9% NaCl (pH 7.4). Lipids were extracted from 100 μL of the LDL suspension with chloroform-methanol (2:1), dried under nitrogen, redissolved in cyclohexane, and analyzed at 234 nm using a spectrophotometer (Shimazu). EDTA was added to the sample to prevent oxidation during sample preparation.

Statistical analysis The data was analyzed using the SPSS package for Windows (Version 10). Values are expressed as the mean±standard error (SE). The data was evaluated using a one-way ANOVA and the differences between the means were assessed using Duncan's multiple range test. The differences were considered significant at $p<0.05$.

Results and Discussion

The groups did not vary significantly in their initial or final body weights, body weight gain, food intake, and the food efficiency ratio (FER) during the experimental period (Table 2). SD supplementation for 10 weeks significantly reduced DMH-induced colonocytic DNA damage (tail intensity: 68.5±2.3% in the DMH group vs. 55.1±3.4% in DMH+SD group), but soybean supplementation alone did not. The comet images of the colonocytes following DMH and DMH+SD treatment are shown in Fig. 3. SD treatment

Table 2. Effects of soybean *dongchunghacho* on the weight gain and food efficiency ratio in rats administrated DMH[1)]

	DMH[2)]	DMH+S	DMH+SD
Final body weight (g)	301.4±5.5[ns]	292.7±7.2	289.1±5.8
Weight gain (g/day)	2.4±0.1[ns]	2.3±0.1	2.3±0.1
Food intake (g/day)	17.5±0.2[ns]	17.2±0.3	17.2±0.3
FER[3)] (%)	13.8±0.2[ns]	13.4±0.5	13.1±0.2

[1)]Values are the mean±SE for 10 animals in each group; ns, not significant.
[2)]DMH, high-fat and low-fiber diet+DMH injected group; DMH+S, high-fat and low-fiber diet+soybean+DMH injected group; DMH+SD, high-fat and low-fiber diet+soybean *dongchunghacho*+DMH injected group.
[3)]Food efficiency ratio.

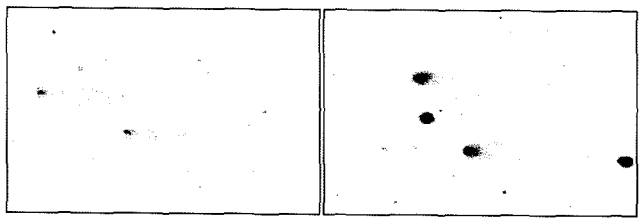

Fig. 3. Fluorescence comet image of rat colonocytes dyed with ethidium bromide (400×). DMH, high-fat and low-fiber diet+DMH injected group; DMH+SD, high-fat and low-fiber diet+soybean *dongchunghacho*+DMH injected group.

also significantly reduced plasma-conjugated dienes, a marker for lipid peroxidation. There were no statistically significant differences in TRAP among the groups.

Animal models of DMH-induced colon cancer are used to evaluate the anticarcinogenic properties of dietary factors by analyzing colonocyte DNA damage (24). DNA damage is the initial step in chemical carcinogenesis; therefore, blocking DNA damage should be the first line of defense against cancer induced by carcinogens (25). Since the comet assay is a supplementary assay of the *in vivo* genotoxicity induced by carcinogen exposure, we investigated the effects of SD on DMH-induced DNA damage in colon cells. SD supplementation effectively attenuated the genotoxic effects of DMH in colon cells. Soybean supplementation alone did not show an effect in colonocyte DNA damage, indicating that the mycelium and spores of *Paecilomyces* spp. are required for this activity.

SD may reduce DNA damage via antioxidant activity. We found that SD reduced plasma lipid peroxidation compared with the DMH or DMH+S control groups. Kwon *et al.* (26) found that diets supplemented with 2% (w/w) powder extracts of *P. tenuipes* fruiting bodies, cultivated in silkworm pupae, lowered plasma and liver thiobarbituric acid reactive substances and liver superoxide dismutase activity in rats, consistent with our results. We previously reported that the administration of 3% *P. tenuipes* increased the plasma total antioxidant potential and decreased the levels of lipid peroxidation in rats fed a high fat-cholesterol diet (27). Extracts of *P. tenuipes* mycelium moderately inhibited monoamine oxidase (MAO), which reduces oxidative stress by limiting the formation of this radical species (28). Partially polymerized, water-soluble-glucans, which are the major physiologically active substances in most mushrooms, may be able to quench free radicals (29, 30), and could produce the antioxidant activity we observed here.

Our results suggest that the consumption of SD could have significant health benefits via the reduction of colonic DNA damage events in rats, as modeled by an early-stage colon carcinogenesis model induced with DMH. Our results also suggest that this effect may be attributed to increased antioxidant activity.

Table 3. Effect of soybean *dongchunghacho* on plasma antioxidant potential and lipid peroxidation in rats administrated DMH[1)]

	DMH[2)]	DMH+S	DMH+SD
Plasma[3)]			
TRAP (mM)	1.25±0.02[ns]	1.26±0.01	1.27±0.02
CD (μM)	25.7±2.7[b4)]	23.4±1.8[b]	18.5±1.8[a]

[1)]Values are the mean±SE for 10 animals in each group; ns, not significant.
[2)]DMH, high-fat and low-fiber diet+DMH injected group; DMH+S, high-fat and low-fiber diet+soybean+DMH injected group; DMH+SD, high-fat and low-fiber diet+soybean *dongchunghacho*+DMH injected group.
[3)]TRAP, total radical trapping antioxidant potential; CD, conjugated dienes.
[4)]Values in the same row that do not share a common superscript are significantly different at $p<0.05$.

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