Inhibition of Formation of Azoxymethane-induced Colonic Aberrant Crypt Foci in Rats by Edible Green Algae Capsosiphon fulvescens and Brown Algae Hizikia fusiforme

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Abstract. Capsosiphon fulvescens (green seaweed) and Hizikia fusiforme (brown seaweed) are marine algae consumed as food supplements, especially in Japan, China and Korea, and are considered traditional medicinal tonics for certain ailments. The aim of this study was to investigate the possible inhibitory effects of dietary C. fulvescens and H. fusiforme on azoxymethane (AOM)-induced colorectal cancer (CRC) in rats. F344 male rats (5 weeks, 150 g) were divided into six groups as follows. Group 1: Injected with normal saline solution and fed control diet (untreated control). Group 2: Injected with AOM and fed control diet (treated control). Group 3: Injected with AOM and fed 1% C. fulvescens diet. Group 4: Injected with AOM and fed 2% C. fulvescens diet. Group 5: Injected with AOM and fed 2% H. fusiforme diet. Group 6: Injected with AOM and fed 6% H. fusiforme diet. Test animals received subcutaneous injections of AOM (15 mg/1 ml/kg body weight) once a week for 2 weeks to induce aberrant crypt foci (ACF) in treated control and experimental groups. We evaluated the effects of dietary C. fulvescens and H. fusiforme at two different dose levels: 1 and 2% C. fulvescens, and 2 and 6% H. fusiforme, on colonic carcinogenesis by AOM in rats. Our results suggest that body weights were not significantly different amongst groups. We found that feeding C. fulvescens and H. fusiforme with a control diet significantly (p<0.05) inhibited the development of ACF in experimental groups. C. fulvescens and H. fusiforme in food also significantly (p<0.05) reduced the proliferating cell nuclear antigen labeling index in the colonic tissues of experimental groups. These results demonstrate the chemopreventive potential of C. fulvescens and H. fusiforme against CRC in an AOM-induced rats.

Colorectal cancer (CRC) is the most common cancer, and a significant cause of death, in developed countries, including the United States (1, 2). Numerous factors including age, physical activity habits, environment, obesity, diabetes, diet, lifestyle, and genetics play important roles in CRC carcinogenesis (3, 4). Many genetic defects/mutations, including the adenomatous polyposis coli (APC) gene, kirsten ras (KRAS) oncogene, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) gene, phosphatase and tensin homolog (PTEN) gene, transforming growth factor (TGFβ) receptor, and tumor protein p53 (TP53) gene, and enzymes such as cyclooxygenase-2 and nitric oxide synthase have been suggested to play vital roles in CRC carcinogenesis (4-6). It is recognized that CRC develops sequentially from polyp to adenocarcinoma. This development may take a long time, as much as 10-17 years (4).

Azoxymethane (AOM) is a tumor-initiating chemical agent commonly used in models of colon cancer (7). Specifically, it can induce the formation of aberrant crypt foci (ACF) by epithelial cells, which then progress to adenomas and malignant adenocarcinomas, that is similar to the pathogenesis of sporadic human colon cancer (8, 9). Thus, it has been extensively used in the study of molecular biology, prevention, and treatment of colon cancer.

ACF are clusters of abnormal tube-like glands on the colonic mucosal surface, and are the earliest morphological changes after treatment with chemical carcinogens such as AOM (10). Evidence strongly suggests that ACF are pre-neoplastic lesions in the colon, and they are recognized as intermediate biomarkers.
of CRC in both rodents and humans (6, 11). Chemically-
induced ACF in rodents have been used to assess new
chemopreventives and diets that might prevent CRC (12).

_Capsosiphon fulvescens_ is a green marine alga consumed
as a food supplement, and is considered to possess medicinal
activity and high nutritional value (13). _Hizikia fusiforme_ is
a brown marine alga, traditionally used as a foodstuff and
medicine in Asia (14). Previous research reported that _C.
fulvescens_ and _H. fusiforme_ exhibited beneficial medicinal
activities, such as antioxidant, anti-diabetic, and antitumor
effects (15).

The purpose of the present study was to identify the
possible inhibitory effect on colon carcinogenesis of feeding
_C. fulvescens_ and _H. fusiforme_ at different dose levels to
F344 male rats. We investigated the effects of _C. fulvescens_
at 1 and 2%, and _H. fusiforme_ at 2 and 6% dose levels in the
rats’ diet. In addition, we immunohistochemically analyzed
the expression of proliferating cell nuclear antigen (PCNA),
which plays a vital role in carcinogenesis.

Materials and Methods

**Chemicals.** All chemicals were of the highest grade and were
purchased from commercial suppliers. AOM was purchased from
Sigma Chemical Co. (St. Louis, MO, USA). Analytical-grade ethyl
ether was purchased from Duksan Pure Chemicals (Ansan, Korea).

**Plant materials and diets.** _C. fulvescens_ and _H. fusiforme_ were
collected from Jeonnam, Wando, South Korea, and were washed
with water and dried. The seaweeds were cut into small pieces and
extracted with 95% ethanol at 80°C for 2 h at 120 rpm, with ethanol
at a ratio of 40 ml/g. The extract was centrifuged (8,000 xg, 10
min), filtered, and concentrated at 60°C. The experimental diets
were prepared by adding 5% brix concentrate to AIN-76A animal
diet (ORIENT BIO Inc, Sangdaewon-Dong, Seongnam-si, South
Korea) (Table I) with 1 or 2% of _C. fulvescens_ and 2 or 6% of _H.
fusiforme_ by dry weight (Table II).

**Animals.** F344 male rats (5 weeks, 150 g) were used in these
experiments (Orient Co Ltd, Seoul, Korea) and maintained in an
environment with controlled temperature (23±2°C) and humidity
(55±7%) under a 12 h light-dark cycle. All animal experiments
were carried out in accordance with the National Institute of Health
guidelines.

**Experimental design.** The rats were divided into six groups for
experimental procedures as follows. In group 1 (untreated control),
saline was injected (1 ml/kg) subcutaneously (sc) during the first
and second weeks of the experimental period. In group 2 (treated
control), AOM was injected (15 mg/1 ml/kg) sc once a week for 2
weeks. The animals in both these groups were fed the control diet
throughout for 11 weeks. Groups 3, 4, 5, and 6 (experimental
groups) were fed experimental diets containing 1 or 2% _C.
fulvescens_ or 2 or 6% _H. fusiforme_, respectively, for 8 weeks
from the third week after the administration of AOM. While group
1 had eight rats, all other groups comprised 10. Body weights and
food intake of the animals were measured throughout the

| Group | Treatment                               | No. of animals |
|------|----------------------------------------|---------------|
| 1    | Saline + control diet                  | 8             |
| 2    | AOM + control diet                     | 10            |
| 3    | AOM + 1% _Capsosiphon fulvescens_ diet | 10            |
| 4    | AOM + 2% _C. fulvescens_ diet          | 10            |
| 5    | AOM + 2% _Hizikia fusiforme_ diet      | 10            |
| 6    | AOM + 6% _H. fusiforme_ diet           | 10            |

AOM: Azoxymethane 15 mg/kg, subcutaneous injection (1 ml/kg).

**Histopathological examination of colonic tumor tissues.** Animals
were sacrificed and colonic tumor tissues were removed
immediately. The tissues were washed with normal saline and fixed
in 10% neutral formalin solution (pH 7.0). After routine paraffin
embedding, 6-8 μm tissue sections were cut, fixed on microscope
slides, and deparaffinized, before being hydrated. Finally, sections
were stained with hematoxylin and eosin (H&E) for histological
examination of colonic cancer specimens.

**Determination of ACF.** The development of carcinoma was
suggested by the detection of ACF, believed to be precancerous
lesions, in colonic mucosa. Rectal crypt foci were excised, and the
colon was spread, washed with potassium phosphate buffer (0.1 M,
pH 7.2), and then fixed in 10% neutral formalin. After fixation and
staining with 2.5% methylene blue, ACF were distinguished
morphologically by their elliptical shape and increased size of
cysts compared to normal crypts and measured microscopically.

**Immunohistochemical evaluation.** PCNA immunohistochemistry
was performed to evaluate the proliferation of tumor cells. Paraffin-
embedded colonic tumor tissues cut into 6-8 μm sections. These were
then immersed in xylene solution to remove paraffin and rehydrate the
tissues while sequentially reducing the ethanol concentration. The
slides prepared in this manner were coated with mouse monoclonal
antibody against PCNA (Santa Cruz Biotechnology, Santa Cruz, CA,
USA), then coated with avidin–biotin peroxidase complex (Zymed

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**Table I. AIN-76A animal diet for controls and experimental groups.**

| Ingredients                                      | g% | kcal% |
|-------------------------------------------------|----|-------|
| Casein. 30 mesh                                  | 200| 800   |
| DL-Methionine                                    | 3  | 12    |
| Corn starch                                      | 150| 600   |
| Sucrose                                          | 500| 2000  |
| Cellulose, BW200                                 | 50 | 0     |
| Corn oil                                         | 50 | 450   |
| Mineral mix S10001                               | 35 | 0     |
| Vitamin mix V10001                               | 10 | 40    |
| Choline bitartrate                               | 2  | 0     |
| Total                                           | 1000 | 3902 |

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**Table II. Experimental animals.**

| Group | Treatment                              | No. of animals |
|------|----------------------------------------|---------------|
| 1    | Saline + control diet                  | 8             |
| 2    | AOM + control diet                     | 10            |
| 3    | AOM + 1% _Capsosiphon fulvescens_ diet | 10            |
| 4    | AOM + 2% _C. fulvescens_ diet          | 10            |
| 5    | AOM + 2% _Hizikia fusiforme_ diet      | 10            |
| 6    | AOM + 6% _H. fusiforme_ diet           | 10            |
feeding the experimental diets containing \textit{C. fulvescens} and \textit{H. fusiforme} did not produce any toxicity. Histological examinations revealed no morphological evidence of fatty liver (data not shown). The body weights of animals in groups 1 and 2 (control groups) were not significantly greater than those in the experimental groups (Table III) that received \textit{s.c.} injections of AOM at 15 mg/kg body weight once a week for 2 weeks to investigate the formation of ACF in the colon. H&E staining revealed significant histopathological changes in the colonic tissues of AOM-treated rats. We found histological abnormalities such as ACF including dysplasia and hyperplasia, abnormally shaped lumens and elongated nuclei in the colonic mucosa of the treated control group (group 2), but these findings were significantly \((p<0.05)\) less frequent in the experimental groups (groups 3-6) (Figure 2). These results demonstrate that \textit{C. fulvescens} and \textit{H. fusiforme} suppress dysplastic cell proliferation in the colonic tissues of AOM-treated rats.

The incidence and number of colorectal tumors after 11 weeks are listed in Table IV. In group 1 (untreated control), no colonic tumors were found. However, group 2 (treated control), colonic tissues were found to contain 58 ACF (Figure 3). In the dose–response study, experimental \textit{C. fulvescens} and \textit{H. fusiforme} di
ti
ds reduced the incidence of ACF formation \((p<0.05)\) in groups 3 and 4, whereas the occurrence of ACF was significantly \((p<0.001)\) lower in groups 5 and 6 compared to the treated control. These findings suggest that \textit{C. fulvescens} and \textit{H. fusiforme} suppress ACF formation induced by AOM in the colon of rats.

To investigate the effects of dietary \textit{C. fulvescens} and \textit{H. fusiforme}, we used immunohistochemistry to analyze the expression of PCNA, a marker of tumor cell proliferation and apoptosis, at week 11. The colonic adenocarcinoma PCNA labeling index was lower \((p<0.05)\) in groups 4 and 5, but in groups 3 and 4 more significantly \((p<0.001)\) lower than in the treated control group (Figure 5).
Discussion

Seaweeds are rich in bioactive substances that may be useful in treating a wide spectrum of diseases. Seaweeds contain health-promoting compounds such as phytochemicals, soluble dietary fibers, lipids, peptides, and minerals that hold potential as dietary supplements and that may reduce the risk of cancer (16, 17). Moreover, macroalgae have been used as food supplements, particularly in Japan, China, and Korea (18). The long life expectancy of Japanese people has been

Table IV. Formation of azoxymethane (AOM)-induced aberrant crypt foci (ACF).

| Group | Treatment                                      | 1    | 2    | 3    | ≥4   | Total   |
|-------|------------------------------------------------|------|------|------|------|---------|
| 1     | Saline + control diet                          | 0    | 0    | 0    | 0    | 0       |
| 2     | AOM + control diet                             | 22.43±2.6 | 11.43±2.3 | 13±3.6 | 11±2.9 | 57.86±2.9 |
| 3     | AOM + 1% Capsosiphon fulvescens diet           | 12.14±1.9 | 5.57±2.0 | 4.29±0.5 | 7.45±1.1 | 29.45±1.4* |
| 4     | AOM + 2% C. fulvescens diet                    | 11.71±1.4 | 6.86±1.3 | 4.14±0.7 | 8.33±0.9 | 31.05±1.1* |
| 5     | AOM + 2% Hizikia fusiforme diet                | 7.22±0.9 | 4.88±0.5 | 4.11±1.3 | 4.97±0.3 | 21.18±0.8** |
| 6     | AOM + 6% H. fusiforme diet                     | 6.57±1.0 | 4.33±0.7 | 3.43±0.9 | 6.17±1.4 | 20.5±1.0** |

AOM given at 15 mg/kg, subcutaneous injection (1 ml/kg). Data are represented as mean±standard error of the mean. Asterisks indicate values significantly different from those in group 2: *p<0.05 and **p<0.01.

Figure 2. Histopathological examination of colonic tissues of F344 rats from group 1, saline + control diet (A); group 2, azoxymethane (AOM) + control diet (B); group 3, AOM + 1% Capsosiphon fulvescens-supplemented diet (C); group 4, AOM + 2% C. fulvescens-supplemented diet (D); group 5, AOM + 2% Hizikia fusiforme-supplemented diet (E); and group 6, AOM + 6% H. fusiforme-supplemented diet (F). Representative microscopic examination was performed with hematoxylin and eosin staining. Histological assessment showed the presence of aberrant crypt foci including dysplasia and hyperplasia, abnormally-shaped lumens and elongated nuclei in the colonic mucosa of the treated control group but a marked reduction in all experimental groups. Sections were examined at 200× magnification.
partly attributed to their dietary habits, including the regular consumption of seaweed (17).

Our experimental data suggest that *C. fulvescens* and *H. fusiforme* are effective chemopreventive agents for CRC. These chemopreventive effects may be due to the high levels of minerals such as Na, Mg, K, Ca, and Fe in both algae (15). AOM has the potential to induce colonic carcinomas in rats by promoting ACF formation. Carcinogenesis is conventionally defined by three stages: initiation, promotion, and progression (19, 20). During the initiation stage, normal cells experience DNA damage (21, 22). During the second stage, promotion, the initiated cells affect normal cells, forming preneoplastic lesions (23). Finally, in the progression stage, malignant tumors develop, leading to metastasis (24).

In the present study, we demonstrated that dietary administration of *C. fulvescens* and *H. fusiforme* caused no significant difference in body weight in groups 3-6 over the experimental period compared with that in groups 1 (untreated control group) and 2 (treated control group). Additionally, there was no significant difference in feed intake between any of the experimental groups. This suggests that *C. fulvescens* and *H. fusiforme* may both have beneficial effects (14) (Table III).

H&E staining of colonic tissues was carried out for histological analyses. Throughout the colonic mucosa of the AOM-treated rats, we observed abnormalities, consistent with molecular aberrations in the treated control group (group 2), but such histological abnormalities were not found in the experimental groups (Figure 2). This demonstrates that dietary *C. fulvescens* and *H. fusiforme* have chemopreventive effects.

The ACF assay evaluates crypts with abnormal morphology, such as those found in group 2. In groups 3-6, dietary administration of *C. fulvescens* and *H. fusiforme* resulted in significantly less ACF formation than in the treated control group (Figure 3), suggesting that these agents counteract the carcinogenic activities of AOM.
Dietary administration of *C. fulvescens* and *H. fusiforme* prevented AOM-induced colorectal proliferative lesions in rats (Figure 4). Experimental groups exhibited pronounced suppression of cell proliferation compared with the treated control group. Groups 3-6 had significantly lower CRC PCNA labeling indices in comparison with that of group 2, suggesting that *C. fulvescens* and *H. fusiforme* might both modulate cell proliferation (Figure 5).

Although the mechanism of inhibition of AOM-induced ACF formation in rats by *C. fulvescens* and *H. fusiforme* has not been established, one possibility is through interference with the cytochrome P450 2E1 (CYP2E1) pathway, due to their potent antioxidant activity (14, 15, 25-27). It has been suggested that suppression of CYP2E1 inhibits chemical carcinogenesis (4, 28). *In vivo*, AOM is largely metabolized by isoform CYP2E1 (4, 29, 30). A previous study established that AOM-induced ACF formation was significantly lower in *Cyp2e1*, knockout mice (29). In the present study, we...
demonstrated that dietary *C. fulvescens* and *H. fusiforme* both suppressed formation of AOM-induced ACF in rats.

In conclusion, many chemopreventive agents have been shown to exhibit inhibitory effects on cancer in preliminary studies. Our results clearly revealed the potential benefits of *C. fulvescens* and *H. fusiforme* at inhibiting ACF formation. However, the optimal dose for an anticancer effect needs to be identified. Further studies on determination of the optimum dose of *C. fulvescens* and *H. fusiforme* in inhibiting ACF formation are required.

**Conflicts of interest**

The Authors declare no conflict of interest in regard to this study.

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