Supplementation of Seaweeds Extracts Suppresses Azoxymethane-induced Aberrant DNA Methylation in Colon and Liver of ICR Mice

So Young Bu¹, Hoonjeong Kwon², Mi-Kyung Sung³

¹Department of Food and Nutrition, Daegu University, Gyeongsan, Korea, ²Department of Food and Nutrition, Seoul National University, Seoul, Korea, ³Department of Food and Nutrition, Sookmyung Women’s University, Seoul, Korea

Background: Seaweedstard and seatangle are commonly consumed seaweeds in Korea and rich sources of non-digestible polysaccharides which possess biological activities. However, anti-mutagenic and anti-cancer activities of these seaweeds under physiological conditions have not been clarified yet. The objective of this study was to investigate the effect of seaweeds consumption on azoxymethane (AOM) induced DNA methylation at N³ and O⁶ position of guanine base, an indicator of DNA damage related to cancer initiation.

Methods: Thirty ICR mice were divided into five groups and fed one of the following diets for two weeks: control diet, diet containing 10% water-soluble or water-insoluble fraction of seaweedstard or seatangle. After two weeks of experimental diet, AOM was injected at 6 hours before sacrifice and N³-methylguanine (N³-meG) and O⁶-methylguanine (O⁶-meG) from the colon and liver DNA were quantified using a gas chromatography-mass spectroscopy.

Results: Water-soluble fractions of both seaweedstard and seatangle significantly reduced AOM-induced production of N³-meG guanine in colon and liver. Also water-soluble fractions of these seaweeds suppressed the level of methylation at O⁶-guanine of colon and liver directly responsible for tumorigenesis. While water-insoluble fraction of seaweedstard suppressed the production of N³-meG in liver this seaweed fraction decreased O⁶-meG and the ratio of O⁶/N³-meG in liver. Water insoluble fraction of seatangle decreased both O⁶- and N³-meG in colon and liver. Supplementation of all seaweeds extracts increased fecal weight of animals and the increase of fecal weight by water-insoluble fraction of seaweeds were higher than that by water-soluble fraction.

Conclusion: Seaweedstard and seatangle intake may effectively prevent colon and liver carcinogenesis by decreasing DNA damage and the mechanism of inhibiting carcinogenesis by seaweeds in a long term study are warranted.

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Key Words: Seaweed, O⁶-methylguanine, N³-methylguanine, Azoxymethane

INTRODUCTION

Numerous experimental and epidemiological studies have indicated that the consumption of certain food, particularly a plant-based food or food component can modulate the risk of cancer. ¹⁻⁵ Seaweedstard (Laminaria japonica) and seatangle (Undaria pinnatifida), belong to brown algae, are commonly consumed seaweeds in Korea and high in bioactive compounds including non-digestible polysaccharides⁷⁻⁸ which have numerous biological activities including anti-inflammatory,⁹ anti-coagulant,¹⁰ anti-viral,¹¹ and anti-angiogenic properties.¹² For example the polysaccharides derived from different Sargassum species attenuate Cyclosporine A-induced oxidative hepatic and renal injury by suppressing oxidative enzyme and by its antioxidative properties.¹³⁻¹⁴ Fucoidan, a type of polysaccharide, exhibits anti-tumor effects by inhibition of cellular proliferation in human lymphoma, melanoma and colon cancer cell lines.⁴⁻¹⁵⁻¹⁷ Also a single gavage of this polysaccharide produces antitumor and anti-metastatic effects in C57BL/6 mice with lung adenocarcinoma.³ Interestingly though the biological function of each...
individual compounds in seaweeds has been well studied in vitro. only a few number of in vivo studies have been conducted to evaluate their biological activities.\textsuperscript{2,3}

Azoxymethane (AOM) is a colon and liver specific carcinogen\textsuperscript{18-21} and used in numerous studies seeking to identify and evaluate potential cancer chemopreventive agents.\textsuperscript{21-24} AOM is metabolically activated in the liver and then delivered to the colon via the blood stream or via bile as glucuronide conjugates. After further activation by Cytochrome P450 CYP2E1 it aberrantly methylates DNA mainly at the N\textsuperscript{7} and O\textsuperscript{6} positions of guanine in DNA.\textsuperscript{10,25} Of these DNA methylation process, methylation at O\textsuperscript{6} position of guanine is more prone to mutagenic and carcinogenic process during DNA replication, through usually a G →A transition mutation in the genes of cell growth or control and lead to the formation of a small benign tumor including aberrant crypt foci which may result in malignant cancer.\textsuperscript{8,20,25-28} Clinical studies with cancer patients also have reported that a high level of DNA adducts in colon are associated with colorectal cancer.\textsuperscript{27,29,30}

Previous studies suggest that the natural anti-cancer agent or food compounds reduce the number of O\textsuperscript{6}-methylguanine (O\textsuperscript{6}-meG) or modulate the expression or activity of transformation enzyme including P450s or β-glucuronidase in advance to suppress precancerous lesion or tumor formation.\textsuperscript{23,24,26} The water extracts of green tea decreased AOM-induced oxidative DNA damage by its antioxidant properties.\textsuperscript{31} In addition significant antimutagenic activity has been reported in the water-soluble extracts of seaweeds or seatangle which showed suppressive effects on the SOS response against DNA damage in Salmonella typhimurium\textsuperscript{32} and the low-molecular weights of non-polysaccharide fraction of seaweeds exhibited a relatively strong antimutagenic N-methyl-N-nitro-N-nitrosoguanidine. chemical precursor of AOM while similar antimutagenic activities were detected in both polysaccharide and non-polysaccharide fractions of seatangle.\textsuperscript{32} On the other hand carrageenan, insoluble polysaccharide reside in both seaweeds and seatangle, rather increased AOM-induced growth of aberrant crypt foci in Fisher344 rats.\textsuperscript{33} These experimental results indicate that water soluble and insoluble fraction of seaweed or seatangle would have dissimilar effects on DNA damage. Hence, in this study, we explored whether the short term supplementation of water soluble and insoluble fraction of seamustard or seatangle in animal diet modifies colon and liver carcinogenesis at the stage of cancer initiation.

**MATERIALS AND METHODS**

1. **Materials**

   AOM, guanase, O\textsuperscript{6}-meG, N\textsuperscript{7}-methylguanine (N\textsuperscript{7}-meG), 8-azaadenine were purchased from Sigma Chemical Co. (St Louis, MO, USA). Other chemicals of reagent grade or higher were purchased from Wako Chemicals (Osaka, Japan).

2. **Extraction of seamustards and seatangles**

   Dried seamustard (Laminaria japonica) and seatangle (Undaria pinnatifida) harvested from Wando were purchased from Korean agricultural cooperative federation in Seoul. Purchased seaweeds were re-dried, minced and blended to a fine powder. Each of dried seaweeds powder were mixed with 1L of distilled water per 20 g of samples and extracted by stirring at 70°C for 6 hours. The water mixture were centrifuged at 200 × g at 4°C for 30 minutes and separated to supernatants (denoted as “water-soluble fraction”) and pellets (denoted as “water-insoluble fraction”) and each fraction of seaweeds were freeze-dried and kept at −20°C until used. The yield of water soluble fraction of seamustard and seatangle were 44.5% and 16.65%, and water insoluble fraction of seamustard and seatangle were 55.5% and 83.3%.

3. **Animals and diet**

   Five to six weeks old male mice (n = 30) were obtained from Animal Experimental Center at Seoul National University. Animals were housed in an environmentally-controlled laboratory (12-hour light/dark cycle at 20°C) upon arrival and allowed to acclimate for 4 days with free access to semi-purified diet and filtered water. After acclimation, mice were weighed and randomly assigned one of the following diets for 2 weeks: control diet, diet containing 10% water-soluble fraction of seamustard or seatangle, diet containing 10% water-insoluble fraction of seamustard or seatangle. Experimental diet composition is shown in Table 1. Food intakes were recorded daily and body weights were measured weekly basis. At the end of each week fecal samples were collected for 48 hours and weighed. Animal protocols in this study were approved by the Animal Care and Use Committee of the Department of Food and Nutrition at Sookmyung Women’s University (Seoul, Korea).
4. Azoxymethane-induced DNA methylation

After two weeks of seaweed supplementation, mice were received intraperitoneal injection of AOM dissolved in saline at a dose of 20 mg/kg of body weight. Mice in control group were injected an equal volume of saline. At 6 hours after AOM treatment mice were sacrificed by CO2 inhalation. The liver and colon were immediately removed and rinsed in ice-cold saline. The liver was weighed and the colon was cut open longitudinally and washed with saline to remove colonic contents. Liver and colon specimens were kept at −80°C until analysis of DNA methylation.

5. DNA hydrolysis and derivatization

Tissue DNA from the liver and colon (mucosa and muscular layer) was isolated by phenol extraction as previously described. The purified sample DNA (200-300 μg) and 8-azaadenine (40 nmol), internal standard for gas chromatography-mass spectrometry analysis were dissolved in DNAase free water and vacuum-dried in vacuum hydrolysis tube (Pierce, Rockford, IL, USA). Dried and powdered DNA were subjected to thermal and acidic hydrolysis by adding 88% formic acid and heating at 145°C for 30 minutes to preferentially release DNA bases. Acid hydrolysates were then re-vacuum-dried and reacted with guanase (4 milliunit/100 μg of DNA) in 20 mM phosphate buffer (pH 8.0) for an hour. Enzyme proteins were removed by centrifugation at 12,000 × g at 4°C for 12 minutes. The dried pellets were purged with pure nitrogen and sealed with short thread caps with Teflon tape (Fisher, Pittsburg, PA, USA). Mixtures of bis (trimethylsilyl) trifluoroacetamide/Acetonitrile (120 μl: 2:1, v/v) were added and subjected to derivatization at 120°C for 30 minutes.

6. Gas chromatography-mass spectrometry analysis of N7-methylguanine and O6-methylguanine

After derivatization, the samples were analyzed on a Hewlett-Packard 5973 mass selective detector interfaced with a Hewlett-Packard 6890 gas chromatograph (Agilent, Santa Clara, CA, USA). The injection port and the gas chromatography-mass spectrometry interface were kept at 200°C and gradually increased up to 300°C by rate of 8°C/min. Separations were performed on a SPB-5 fused silica capillary column (length 50 m and internal diameter 0.2 mm) coated with cross-linked 5% phenylmethyl-siloxane (film thickness, 0.33 μm). Helium was used as a carrier gas with a flow rate of 1 ml/min. Mass spectrum scan for each standard compound was performed during assay condition development. After observation of the major ions for each compound, selective ion monitoring (m/z 309 and 294 for N7-meG and O6-meG; m/z 265 and 280 for 8-azaadenine) was performed.

7. Statistical analysis

The data were analyzed by one-way analysis of variance with the SAS program (SAS 9.0, SAS Institute Inc., Cary, NC, USA). When the F-values were significant by analysis of variance, post hoc analysis using Fisher’s least squares means separation test was used to determine differences between experimental groups. Data are presented as the mean ± standard deviation with P < 0.05 being statistically significant.
So Young Bu, et al: Seaweeds Suppress AOM-induced DNA Methylation

Table 2. Food intake and weight gain of animals fed different experimental diets

| Group           | Food intake (g/day) | FER a | Weight gain         |
|-----------------|--------------------|-------|---------------------|
| Control         | 7.14 ± 0.10 c      | 1.53 ± 0.24 b | 4.88 ± 0.90 b  |
| Seamustard-S3   | 7.86 ± 0.00 b      | 1.43 ± 0.22 b | 4.21 ± 2.11 b   |
| Seamustard-IS4  | 6.21 ± 0.27 c      | 1.81 ± 0.28 b | 5.97 ± 1.48 b   |
| Seatangle-S5    | 8.23 ± 0.15 c      | 1.37 ± 0.21 b | 4.42 ± 2.22 b   |
| Seatangle-IS6   | 8.20 ± 0.14 c      | 1.38 ± 0.24 b | 4.76 ± 1.49 b   |

1) Food efficiency ratio (FER) = weight gain (g)/food intake (g).
2) Means ± standard deviation (n = 6 per group).
3) Water soluble (S) fraction of Seamustard.
4) Water insoluble (IS) fraction of Seamustard.
5) Water soluble fraction of Seatangle.
6) Water insoluble fraction of Seatangle.

Table 3. Fecal weights of experimental animals at week 1 and week 2

| Group           | Week 1 (g) b | Week 2 (g) b |
|-----------------|--------------|--------------|
| Control         | 0.79 ± 0.12 dx| 2.41 ± 0.33 cx |
| Seamustard-S3   | 1.68 ± 0.24 bc| 2.77 ± 0.39 bc |
| Seamustard-IS4  | 2.36 ± 0.53 ab**| 3.62 ± 0.25 ab** |
| Seatangle-S5    | 1.41 ± 0.24 bc| 2.59 ± 0.43 bc |
| Seatangle-IS6   | 2.22 ± 1.06 ab**| 4.16 ± 0.26 ab** |

1) Wet weights of fecal collected for 48 hours. 2) Data are means ± standard deviation (n = 6 per group).
3) Water soluble (S) fraction of Seamustard.
4) Water insoluble (IS) fraction of Seamustard.

Table 4. Effect of water-soluble and -insoluble fraction of seatangle and seamustard on azoxymethane (AOM)-induced colon DNA methylation

| Group           | N7-meG /DNA (fmol/µg) | O6-meG /DNA (fmol/µg) | N7-meG/O6-meG |
|-----------------|-----------------------|-----------------------|---------------|
| Control         | 1837.52 ± 532.25 a    | 283.32 ± 4.11 a       | 0.15          |
| Seamustard-S3   | 732.01 ± 183.93 b     | 75.46 ± 87.89 b*      | 0.10          |
| Seamustard-IS3  | 1148.67 ± 223.82 b    | 54.48 ± 22.73 b**     | 0.05          |
| Seatangle-S5    | 1085.49 ± 327.17 b    | 10.66 ± 7.33 b**      | 0.01          |
| Seatangle-IS5   | 936.45 ± 80.45 b      | 50.51 ± 26.14 b**     | 0.05          |

1) Data are means ± standard deviation (n = 6 per group). 2) Water soluble (S) fraction of Seamustard.
3) Water insoluble (IS) fraction of Seamustard.

RESULTS

1. Food intake and body weight

Daily food intakes of seamustard and seatangle group were significantly higher than that of the control group while food efficiency ratio which reflects the contribution of food intake to body growth is not statistically different in all diet groups (Table 2). There was no statistical difference of body weight gain between control and experimental diet groups (Table 2). Liver weight or relative liver weight to body weight was not significantly different among all groups (data not shown).

2. Fecal weight

To investigate if water-soluble or -insoluble fraction of seaweeds affects fecal content and weight, feces of animals from each diet group were collected for 48 hours and weighed. Overall fecal weights of animals fed seaweeds diet were higher than that of control groups (Table 3). Particularly the supplementation of water insoluble fraction of both seamustard and seatangle substantially increased fecal weight of animals (up to 190%, \( P < 0.01 \)) compared to soluble fraction of seaweeds diet and control diet.

3. DNA adduct formation

AOM-induced N7- and O6-guanine methylation of colon were significantly decreased (37-60%) in animals fed diets containing both water-soluble and insoluble fractions of seamustard or seatangle compared to those in animals fed control diet (Table 4 and 5). The reduction of O6-meG formation in colon by water soluble and insoluble fraction of seaweeds was in range of 73-96% (Table 4). In liver the level of N7-meG and O6-meG were deceased in water soluble and insoluble fraction of seamustard and water soluble fraction of seatangle diet group compared to those in control diet group (Table 5). Although only the water-insoluble fraction of seatangle fed group showed significantly higher level of N7-meG than that of the other diet groups, the level of O6-meG
was lower than that of control diet group which generate ratio of $O^6$-$N^7$-meG down to 0.02 in water-insoluble fraction of seatangle diet group (vs. 0.1 in control group). In addition the higher extent of suppression in AOM-induced DNA methylation were shown in liver of animals fed soluble fraction of seaweeds than in animals fed insoluble fraction of seaweeds.

**DISCUSSION**

Findings from this study which show that the supplementation of water soluble and insoluble fraction of seaweeds and seatangle decreases DNA methylation more prominently at $O^6$ position of guanine versus $N^7$ position of guanine indicate the presence of functional compounds in seaweeds and their potent anti-cancer activity. Though many research findings are available on the medicinal bioactive substances from natural food sources which may help to reduce cancer risk, few studies have reported anticancer activity of seaweeds or their bioactive components in animal model. Hence this preliminary study contributes to augment physiological evidence of anti-carcinogenic properties of seaweeds.

Of many nutrients and bioactive compounds in seaweeds and seatangle non-digestible polysaccharides has been well established and there are several surrogate evidences and theories that these components of seaweed help to attenuate carcinogenesis. For example regular consumption of dietary fiber/polysaccharides changes the composition of the human gut microflora and on bowel habits under systemic insulin and hormonal exposures. In particular water insoluble polysaccharides are associated with the increase of fecal bulk and decreased contact of enterocytes with carcinogens while water-soluble polysaccharides are largely fermented by colonic bacteria and induce the production of short-chain fatty acids which reduce the risk of malignant tumor. These well-known findings support our current results that the seaweeds extracts increase fecal weights and water-insoluble fraction of seaweeds has more pronounced effects on fecal weights than water-soluble fraction implicating a possible mechanism how these seaweeds decreased the production of $N^7$-meG and $O^6$-meG. Other than their effects on gastrointestinal tract the role of polysaccharides, the presence of other bioactive components such as antioxidants and hepatic immune-modulators in seaweeds support our findings of decreased production of $N^7$-meG and $O^6$-meG by seaweed extracts in liver. For example black tea which possesses high polyphenols significantly suppresses AOM-induced production of $N^7$-meG and $O^6$-meG in liver. Also lemon grass extracts substantially decrease the production of $O^6$-meG in liver by its antioxidants activity (more than 50% inhibition of thiobarbituric acid-reactive substances). Furthermore our current findings that the level of suppression in $O^6$-meG production in liver by water-soluble fraction of seaweeds was two-fold higher than that by water-insoluble fraction of seaweeds indicate that distinguished hepatic actions of bioactive compounds between water-soluble and -insoluble fraction of these seaweeds.

Table 5. Effect of water-soluble and insoluble fraction of seatangle and seamustard on AOM-induced liver DNA methylation

|                  | $N^7$-meG (fmol/μg) | $O^6$-meG (fmol/μg) | $N^7$-meG (fmol/μg) |
|------------------|---------------------|--------------------|---------------------|
| Control          | 3303.86 ± 312.83    | 87.03              | 0.11                |
| Seamustard        | 1282.69 ± 521.69    | 87.03              | 0.07                |
| Seatangle         | 2009.23 ± 911.34    | 89.01              | 0.04                |
| Seatangle-S       | 6331.64 ± 877.02    | 149.01             | 0.02                |

$^{a}$Data are means ± standard deviation (n = 6 per group). $^{b}$Water soluble (S) fraction of Seamustard. $^{c}$Water insoluble (I) fraction of Seamustard. $^{d}$Water soluble fraction of Seatangle. $^{e}$Water insoluble fraction of Seatangle. $^{*}P < 0.01$. $^{**}P < 0.001$. a,b,c Means with different letters within a column are significantly different from each other at P < 0.05 as determined by Fisher’s least significantly different test. $N^7$-meG, $N^7$-methylguanine; $O^6$-meG, $O^6$-methylguanine.
noids and polyphenols and suppress AOM-induced DNA damage and cancer progression by decreasing oxidative stresses in Sprague-Dawley rats.\(^\text{41}\) Additionally lemon grass extracts high in antioxidants substantially suppress AOM-induced \(\beta\)-glucuronidase activity, phase II enzyme and decrease aberrant crypt foci production. Along with prominent effect on the production of \(\text{O}^6\)-me\(G\) than \(\text{N}^7\)-me\(G\) by seaweeds in our study, in vitro anti-mutagenic effects against AOM precursor compound\(^\text{32}\) and the presence of several bioactive compounds other than polysaccharides in seaweeds implicate that transformation of carcinogen or co-factors boosting carcinogenesis such as physiological anti-oxidant depletion in both colon and liver might be affected by seaweeds. Therefore a long term effect of seaweed diet under chronological exposure of AOM which considers timely sufficient DNA repair processes, several transformation enzymes, and antioxidative enzymes along with neoplastic legion development will clarify the mechanism how these seaweeds suppress DNA damage and exert anticancer activity.

As a form of diet or whole food the anticancer properties of seaweeds in experimental study has not been well characterized but a few numbers of clinical and epidemiological studies suggest that daily intake of seaweeds have beneficial effect in several types of cancers.\(^\text{1,2,6-8}\) For instance in Japan people who regularly eat seaweeds have dramatically lower rate of breast cancer.\(^\text{1}\)

Recently a clinical study in Japan showed that intake of dietary seaweeds lowered the level urokinase-type plasminogen activator receptor involved in angiogenesis in cancer survivors.\(^\text{2}\) Also the frequent consumption of seaweed is associated with the reduction of colorectal cancer (\(\chi^2 = -0.67\), odds ratio = 0.80, 95% confidence interval = 0.50-1.27).\(^\text{43}\) Although seaweeds consumption effectively suppressed DNA methylation in current study, each fraction of seaweeds also contain other nutritive or non-nutritive components, and does not solely represent polysaccharides found in seaweeds. It is unclear at this point whether the effects on DNA methylation in colon and liver reported here are the results of an individual polysaccharides or the action of the polysaccharides as a whole. Indeed, it has been reported that intake of minerals and vitamins are associated with a decreased risk of colon cancer, in a meta-analysis.\(^\text{44}\) In their analysis of thirteen cohort studies they found that multivitamin supplementation had a protective effect against cancer (odds ratio = 0.88. 95% confidence interval = 0.81-0.96). So the presence of minerals and vitamin in seaweed cannot be ignored in assessing the cancer protective effect of seaweed consumption.

Overall this study suggests the possibility of anti-carcinogenic effect of seaweeds supplementation by showing reduced DNA methylation at \(\text{O}^6\)-position of guanine which prone to inducing carcinogenesis in colon and liver in conjunction with increased fecal weight. Anchored in positive results from this study, chronological effects of seaweeds on cancer progression and mechanism how seaweeds restrain carcinogenesis should be evaluated in future study. Simultaneously the relationship between seaweeds consumption and cancer development in clinical study or epidemiological evidence need to be further investigated.

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