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Original Article

Dietary Oyster (*Crassostrea gigas*) Extract Increases the Goblet Cell Counts in the Ileum of Mice

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Summary

We previously reported that dietary oyster (*Crassostrea gigas*) extract (OE) promoted fecal mucin excretion in mice fed a normal-fat (NF) diet. However, it remains unclear whether OE increases fecal mucin excretion and ileal goblet cell counts in mice fed a high-fat (HF) diet. This study examined the effect of OE intake on fecal mucin excretion and ileal goblet cell counts in mice fed NF and HF diets. Male C57BL/6 J mice were divided into four groups and fed an AIN-93G diet (NF diet), AIN-93G modified HF diet (HF diet), NF diet containing 5% (w/w) OE (NFOE diet), and HF diet containing 5% (w/w) OE (HFOE diet). After 28 days of experimental diet feeding, feces and distal ileum were collected. Fecal mucin content was quantified using a fluorometric assay kit that discriminates *O*-linked glycoproteins from *N*-linked glycoproteins. Goblet cells in the villi of the distal ileum were counted via staining with Alcian blue-periodate Schiff stain. Fecal mucin content and ileal goblet cell count were significantly increased by OE intake ($p = 0.013$ and 0.007, respectively). OE intake may enhance the intestinal barrier function by increasing the number of goblet cells and mucin secretion in the distal ileum.

We investigated the nutritional and health-promoting functions of oyster extract (OE) prepared by extracting and concentrating the nutritional and functional components of oyster (*Crassostrea gigas*) meat. We have reported the bioavailability of zinc[1,2)], hepatoprotective effect[3)], prevention of colorectal precancerous lesions[4,5)], and prevention of renal damage[6)] as the nutritional and health-promoting functions of OE intake. Regarding the gut environment, which has been actively studied in relation to health in recent years, OE has proven effective in increasing the proportion of *Lactobacillus* genus in the feces of rats and mice[7,8)]. In addition, OE attenuates experimental colitis symptoms induced by dextran sulfate sodium, partly owing to improvements in the gut microbiota and short-chain fatty acid composition[9,10)]. Because the gut is exposed to pathogenic bacteria and their toxins, it has a robust barrier function and immune system to protect the body from these factors[11)]. Mucin is a key component of the gut barrier that prevents the entry of pathogenic bacteria and their toxins[12,13)]. Dietary OE enhances fecal mucin excretion in mice fed a normal-fat (NF) diet[14)]. However, it remains unclear whether the addition of OE to a high-fat (HF) diet used to induce obesity and hyperlipidemia in mice enhances fecal mucin excretion. In contrast, mucin is produced and secreted by goblet cells and scattered among absorptive epithelial cells. The number of goblet cells is affected by dietary components[15)]. However, the effect of OE on the number of ileal goblet cells has not yet been investigated. In this study, we fed mice two experimental diets (NF and HF) supplemented with OE and evaluated the effects of OE on fecal mucin excretion and ileal goblet cell counts.

Materials and Methods

Prepared the experimental diets

The OE was supplied by Japan Clinic Co., Ltd. (Kyoto, Japan). The nutritional compositions of OE were 50.6 g/100 g carbohydrate, 28.1 g/100 g crude protein, 2.0 g/100 g crude fat, 3.9 g/100 g moisture, 15.4 g/100 g ash, and 8.24 g/100 g of NaCl. The experimental diets were prepared as an NF diet (soybean oil 7%, w/w) with modified NaCl content in the AIN-93G composition[16)] and an HF diet, in which the NF diet was modified to 20% higher fat (soybean oil 7% + lard 13%, w/w). In addition,

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5% (w/w) OE was added to the NF and HF diets, and the NFOE and HFOE diets were prepared such that the carbohydrate, protein, fat, and NaCl contents were equivalent to those of the NF or HF diets. The composition of each diet was the same as a previous study[8].

Animal experiment

This study was conducted with the approval of the Kansai University Animal Experiment Committee (Approval No. 2317). Four-week-old male C57BL/6 J mice (Japan SLC Inc., Shizuoka, Japan) were used as experimental animals. After a 7-day acclimation period to the AIN-93G diet, 28 mice were divided into four groups of seven mice each with equal average body weight (BW). Mice were housed at a room temperature of 22 ± 1℃ with a 12-h light/dark cycle (8:00–20:00). The mice had unrestricted access to food and water. Food efficiency was calculated by dividing BW gain by food intake. After 28 days of experimental diet feeding, mice were euthanized under isoflurane anesthesia (Kyoritsu Seiyaku, Tokyo, Japan), and the liver, spleen, kidney, distal ileum, and white adipose tissue (WAT) were collected and weighed. The distal ileum was washed with cold saline and immersed in Carnoy's solution. One-day old feces from the day before dissection were collected, weighed, flash-frozen in liquid nitrogen, and stored at −80℃.

Analysis of fecal mucin content

The feces were freeze-dried and powdered using a conventional mill. Fecal mucin content was measured using a Fecal Mucin Assay Kit (CosmoBio Co. Ltd., Tokyo, Japan) according to the manufacturer's protocol.

Histological analysis

The distal ileum was kept in Carnoy's solution for 3 h and then placed in phosphate-buffered saline (PBS) for 24 h, followed by 70% ethanol for 24 h at 22 ± 2℃. The samples were transferred to fresh tubes with 70% ethanol and stored at 4℃ until embedded in paraffin. The paraffin-embedded tissues (n = 5) were sectioned (4 μm) using a microtome and adhered to glass slides. The distal ileum was stained with Alcian blue periodic Schiff (AB-PAS) according to the standard protocol to observe the histological structure of the villi and goblet cells. The stained tissues were scanned using a Nanozoomer2.0HT (Hamamatsu Photonics K.K., Hamamatsu, Japan). In the AB-PAS-stained sections, the number of goblet cells per villus was counted using the NDP.view2 software (Hamamatsu Photonics K.K.).

Statistical analysis

The data are presented as the mean ± standard error. Two-way analysis of variance (ANOVA) was performed with fat content in the experimental diet and the presence or absence of OE in the experimental diet as factors. Statistical significance and statistical tendency were set at $p < 0.05$ and $0.05 \leq p < 0.10$, respectively. If a significant difference in the interaction was found in the two-way ANOVA, a multiple group comparison was conducted using Tukey's multiple comparison test. Statistical analyses were performed using GraphPad Prism ver. 7.0d (GraphPad Software, California, USA).

Results and Discussion

The growth parameters and organ weights are shown in **Table 1**. Trends were observed for food intake and efficiency, which were influenced by the fat content in the experimental diets ($p = 0.079$ and 0.071, respectively). No significant differences in the final BW were observed between the groups. In contrast, the HF diet increased the epididymal and inguinal WAT weights ($p = 0.015$ and 0.005, respectively). Furthermore, the epididymal WAT weight in the HF group was significantly higher than that in the NF group. The mesenteric WAT weight tended to increase with the HF diet ($p = 0.087$). Long-term feeding of C57BL/6 J mice with an HF diet increases BW and induces obesity compared to a low-fat diet[17]. In this study, we determined that the HF diets-fed mice were slightly obese, as the epididymal and inguinal WAT weights increased significantly, and the mesenteric WAT weight also showed a tendency to increase.

Fecal mucin excretion was analyzed to estimate the effect of OE intake on the gut barrier function, (**Fig. 1**). Fecal mucin excretion was significantly decreased by HF diet ($p = 0.006$) and increased by OE intake ($p = 0.013$). Our previous study showed that dietary OE enhanced fecal mucin excretion in mice fed an NF diet[14]. Dietary OE increased fecal mucin excretion not only in mice fed the NF diet but also in mice fed the HF diet, which decreased fecal mucin excretion.

The distal ileum sections were evaluated for goblet cell counts by highlighting the goblet cell population using AB-PAS staining. Representative images of AB-PAS staining of the distal ileum are shown in **Fig. 2 A**. The number of ileal goblet cells, shown in **Fig. 2B**, was significantly increased by OE intake ($p = 0.007$), but the fat content of the experimental diets had no effect ($p = 0.196$). Goblet cells play a critical role in maintaining intestinal homeostasis by producing the key components of the mucus lay-

Table 1 Growth parameters and organs weights

	Experimental groups				p value of two-way ANOVA		
	NF	NFOE	HF	HFOE	OE	Fat content	Interaction
Growth parameters							
Initial BW (g)	16.0 ± 0.2	15.9 ± 0.3	16.0 ± 0.5	16.0 ± 0.4	0.968	0.968	0.968
Final BW (g)	21.5 ± 0.3	22.7 ± 0.5	22.9 ± 0.5	22.5 ± 0.6	0.393	0.215	0.131
BW gain (g/day)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.470	0.319	0.213
Food intake (g/day)	2.5 ± 0.1	2.6 ± 0.1	2.4 ± 0.1	2.5 ± 0.1	0.100	0.079	0.551
Food efficiency (g/g)	0.07 ± 0.00	0.08 ± 0.00	0.09 ± 0.01	0.09 ± 0.01	0.816	0.071	0.168
Organ weights (g/100 g BW)							
Liver	3.38 ± 0.19	3.35 ± 0.21	3.33 ± 0.13	3.39 ± 0.14	0.927	0.983	0.827
Spleen	0.28 ± 0.02	0.31 ± 0.02	0.29 ± 0.02	0.32 ± 0.02	0.281	0.631	0.835
Kidney	1.10 ± 0.06	1.19 ± 0.05	1.24 ± 0.04	1.21 ± 0.05	0.514	0.164	0.291
Epididymal WAT	1.53 ± 0.08 [a]	1.90 ± 0.15 [ab]	2.24 ± 0.16 [b]	1.95 ± 0.13 [ab]	0.787	0.015	0.034
Mesenterial WAT	0.45 ± 0.04	0.59 ± 0.07	0.67 ± 0.08	0.61 ± 0.05	0.532	0.087	0.155
Perirenal WAT	1.25 ± 0.10	1.53 ± 0.11	1.54 ± 0.13	1.65 ± 0.16	0.166	0.147	0.543
Inguinal WAT	0.97 ± 0.09	1.27 ± 0.07	1.49 ± 0.16	1.48 ± 0.10	0.235	0.005	0.197

Values are the means with standard error shown by vertical bars based on seven measurements.
When an interaction was significant using two-way ANOVA ($p < 0.05$), Tukey multiple range test was performed; means in the same row not sharing a common superscript differ significantly ($p < 0.05$) in Tukey multiple range test.
ANOVA, analysis of variance; BW, body weight; HF, high-fat diet; NF, normal fat; OE, oyster extract; WAT, white adipose tissue.

er[18]. A previous study showed an association between mucin secretion in the small intestine and goblet cell count upon dietary fiber intake[19]. Therefore, increased goblet cell counts due to OE intake may be associated with increased fecal mucin excretion via increased mucin secretion in the ileum.

In mice with knocked out *Muc2* gene, which is prominently expressed in goblet cells, inflammation is induced due to direct bacterial contact with intestinal epithelial cells and eventually spontaneously develop colon cancer[20]. In contrast, transgenic mice with enhanced mucin production have a more solid mucin layer[21]. Mucin is used to form the only large physical barrier that prevents the invasion of intestinal epithelial cells by intestinal pathogens, bacteria, and viruses[22]. Therefore, we hypothesized that the increased fecal mucin excretion and the number of ileal goblet cells observed in mice fed OE-containing diets would lead to increased intestinal barrier function. A previous study showed that the probiotic *Lactobacillus plantarum* HNU082 optimized intestinal barrier function by increasing goblet cell counts and *Muc2* gene expression in ulcerative colitis mice[23]. We previously reported that OE increases the relative proportion of the *Lactobacillus* genus in the feces of rats and mice[7,8]. Therefore, the increase in ileal goblet cell count following OE intake may be associated with an increase in the relative proportion of the *Lactobacillus* genus. Further studies are needed to elucidate the mechanism by which OE intake increases ileal goblet cell counts.

In conclusion, these results suggest that dietary OE strengthens intestinal barrier function by increasing goblet cell counts in the distal ileum and promoting mucin secretion in mice fed NF and HF diets.

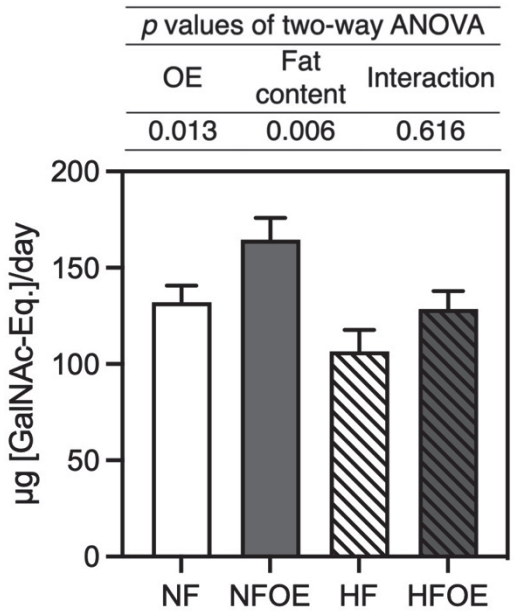

Fig. 1 Oyster-extract intakes increased fecal mucin excretion.
Values are the means with standard errors shown as vertical bars based on seven measurements. Statistical analysis was performed using a two-way ANOVA.
ANOVA, analysis of variance; GalNAc, *N*-Acetylgalactosamine; HF, high fat; NF, normal fat; OE, oyster extract.

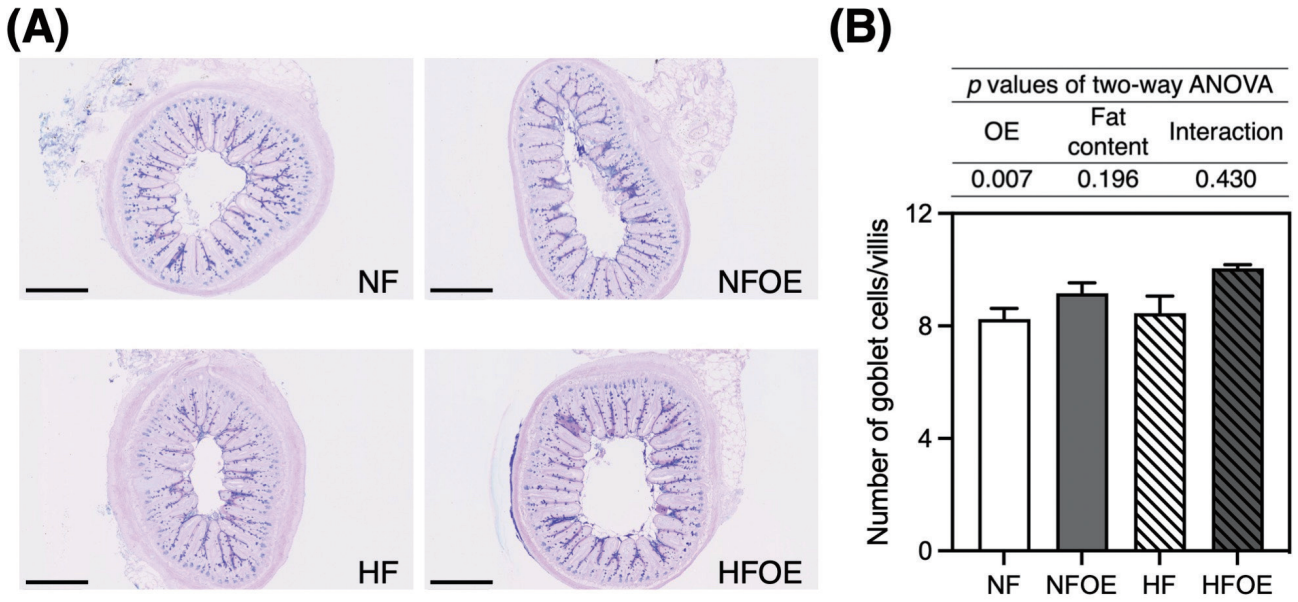

Fig. 2 Oyster-extract intakes increased the number of goblet cells in the distal ileum region.
(A) Alcian blue staining of the distal ileum. Scale bar denotes 250 μm. (B) Goblet cells per villus were counted.
Values are the means with standard errors shown as vertical bars based on five measurements. Statistical analysis was performed using a two-way ANOVA.
ANOVA, analysis of variance; HF, high-fat diet; NF, normal fat; OE, oyster extract.

Author Contributions

TI, HM, YM and RH designed the study. TO, TI, and RH participated in data collection. TO, TI, and RH analyzed the data. RH, MY and KF drafted the manuscript. All authors contributed to the revision and preparation of the final version of the manuscript. All authors approved the final version of the manuscript.

Conflicts of Interest

TI, HM, and YM are employees of the Japan Clinic Co., Ltd., who supplied the oyster extract and funded the study. RH and MY received donations from Japan Clinic Co., Ltd. The authors declare no conflict of interest.

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