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Dietary squid ink polysaccharide defenses goblet cells to protect small intestine from chemotherapy induced injury

Tao Zuo\textsuperscript{a}, Lu Cao\textsuperscript{a}, Changhu Xue\textsuperscript{a}, Qing-Juan Tang\textsuperscript{a}* 

\textit{a. College of Food Science and Engineering, Ocean University of China, Yushan Road 5\textsuperscript{th}, Qingdao, Shandong province, PR. China, 266003}

Author for correspondence: Qing-Juan Tang, MD, Associate Professor, College of Food Science and Engineering, Ocean University of China, Add: 5 Yushan Road, Qingdao, Shandong Province, China, Postcode: 266003 Tel: +86-532-82032597, e-mail: tangqingjuan@ouc.edu.cn, ouczt@hotmail.com

\* Author for correspondence. Phone: +86-532-82032597. Fax: +86-532-82032468. E-mail: tangqingjuan@ouc.edu.cn, ouczt@hotmail.com
Abstract: Gastrointestinal mucositis induced by chemotherapy is associated with alterations of intestinal barrier function due to the potential damage induced by anti-cancer drugs on the epithelial cells. Goblet cell, an important epithelial lineage in the intestine, contributes to innate immunity by secreting mucin glycoproteins. Employing a mouse model of chemotherapy induced intestinal mucosal immunity injury by cyclophosphamide, we demonstrated for the first time that polysaccharide from the ink of *Ommastrephes bartrami* (OBP) enhanced Cyto18, mucin expression in goblet cells. The up-regulation of mucins by OBP relied on the augmented quantity of goblet cells, but not on the changes in endoplasmic reticulum (ER) ultrastructure. Our results may have important implications for enhanced immunopotentiation function of functional OBP on intestinal mucosal immunity against intestinal disorders involving inflammation and infection.

Key words: squid ink, polysaccharide, goblet cells, mucin
1. Introduction

Mucosal barriers are endowed with multi-functional defense mechanisms that selectively handle harmful or innocuous antigens to ensure local homeostasis. The gastrointestinal tract, which is part of the mucosal system, is exposed to a multitude of ingested or inhaled microorganisms, environmental and food antigens. In particular, the vulnerable intestinal mucosa is persistently exposed to potentially harmful ingested agents. Various clinical studies have shown that chemotherapy could damage host immunity system, and cause intestinal microflora imbalance. Infection is one of the most commonly encountered complications during chemotherapy treatment, resulting in gastrointestinal disorders, such as persistent diarrhea, stomachache, emesis, and bacterial systemic dissemination. That is extremely painful for chemotherapeutic patients, which make it urgent and imperative to find a cure to reduce their pains. Because of the side-effects of pharmaceutical products, functional food or its natural bioactive components become a relatively good choice for chemotherapeutic patients.

The goblet cell, which is one of four major epithelial cell lineages in the small intestine, is part of the first-line protection of the mucosal surface that belongs to the innate mucosal immune system for the host’s defense against possible pathogens. Gastrointestinal epithelium is covered with protective mucus composed predominantly of mucin glycoproteins which are synthesized and secreted by goblet cells. The mucin glycoproteins act as a medium for lubrication, protection between the luminal contents and the epithelial lining, and more importantly, preventing gut bacteria penetrating the epithelium barrier. Recent evidence revealed that goblet cells deliver luminal antigen to CD103+ dendritic cells in the small
intestine, which gives us another understanding of how pathogen-specific immunity is elicited while avoiding inappropriate responses to the background of innocuous antigens. The proper functions of goblet cells are essential for treating against intestinal infections and inflammatory diseases.

Chemotherapeutic cyclophosphamide (Cy) is an anti-tumor drug with a wide spectrum of clinical uses and it has been proved to be effective in the treatment of cancer and nonmalignant disease states such as rheumatoid arthritis. However, high doses of anti-cancer drugs can damage the intestinal mucosa which can lead to clinical problems such as bacterial translocation, diarrhea and dyskinesia. The complications of anti-cancer chemotherapy include gastrointestinal (GI) mucositis, which represents injury of the rest of the alimentary tract beyond oral mucositis. This condition is most prominent in the small intestine, but it also occurs in the esophagus, stomach, and large intestine. GI mucositis induced by chemotherapy is associated with alterations of intestinal barrier function due to the potential damage induced by the anti-cancer drugs on the epithelial cells of the intestinal mucosa. Cytotoxic drugs impair the turn-over of intestinal epithelia, induce flattening of the villi and increase the exposure of luminal contents to crypts. In this process, goblet cells which belong to intestinal epithelia, will be under attack by cyclophosphamide.

Previous studies have been focused on the function of goblet cells and their relationship with dysfunction of intestinal disease. However, few studies pertaining to the effects of food-sourced substances or chemicals on mucosal related goblet cells have been reported. As food is intimately connected with intestinal epithelia, it has tremendous opportunities to regulate the activity of goblet cells and exerts a positive influence on mucosal innate
immunity.

Tremendous studies have been focused on the immunomodulatory and other bioactivity functions of functional polysaccharide, such as, the immune protective activities of *Ficus carica* polysaccharide and *Basella rubra* polysaccharide \(^{18,19}\), the anti-tumor activities of *Tricholoma matsutake* polysaccharide and *Angelica sinensis* polysaccharide \(^{20,21}\). However, few studies pertaining to the effects of foodborne polysaccharide on intestinal mucosal immunity were reported, even none on goblet cell. Furthermore, studies of marine-derived squid ink, which has little commercial use and is usually discarded, have focused on its anti-tumor \(^{22}\), anti-oxidant\(^{23}\), and anticoagulant activities \(^{24}\). The squid ink polysaccharide OBP could interact with intestinal epithelial cells, including goblet cells, which makes it possible to regulate goblet cells response directly and rapidly. Also, in our previous study we showed OBP promoted intestinal SIgA secretion and ameliorated intestinal microbiota homeostasis \(^{25,26}\). Furthermore, whether OBP could regulate goblet cells and mucin secretion need to be further studied. Thus, the aim of the present study is to assess the effect of the polysaccharide from the ink of *Ommastrephes bartrami* (OBP), on promoting the mucin secretion from goblet cells, to prevent pathogens from penetrating or colonizing the intestinal mucosa, and hence improve mucosal immunity, in cyclophosphamide (Cy) induced immunosuppressed mice. This study will further elucidate the mucosal immunity enhancement function of squid ink polysaccharide OBP.

2. Materials and Methods

2.1 Materials
The ink sac of the squid, *Ommastrephes bartrami*, was obtained from Zhou-Shan Fishery Company (Zhejiang, China) and stored at −20 °C before use. TRIzol reagent was obtained from Invitrogen (Carlsbad, USA). M-MLV reverse transcriptase was obtained from Promega (Madison, WI). Maxima SYBR Green qPCR Master mix was purchased from Fermentas (Glen Burnie, Maryland). Cyclophosphamide was purchased from Jiangsu Hengrui Medicine Co., Ltd (Jiangsu, China).

All the chemical reagents used in the experiment were of analytically purity.

2.2 Preparation of OBP

OBP was prepared as previously reported by Chen et al. Briefly, after squid ink of *Ommastrephes bartrami* was acidified to pH 4–5 with 0.1 M HCl and the solution stood for 24 h at 4°C to precipitate melanin, melanin was removed by centrifugation at 5,000 × g for 1 h. Then melanin-free ink was digested with 2 volumes of 1% (w/v) papain in Tris–HCl buffer (50mM, pH 6.8) containing 5mM Cys and 5mM EDTA at 60°C for 24 h. Digestion was repeated twice to ensure the cleavage of the protein/peptide moiety. Melanin-free polysaccharide was obtained after precipitation with 4 volumes of ethanol. The resultant OBP extract was dialyzed against several changes of water and then lyophilized.

2.3 Animals

Male Balb/c mice weighting 18 to 22 g were purchased from Vital River Laboratory Animal Center (Beijing, China). The mice were housed throughout the feeding experiment in a room maintained at a 12 hours light/dark cycle, a constant temperature of 24°C, and a
relative humidity of 65±15%. After a 7 days adaptation period, mice were assigned to five
groups with 10 mice each group: Normal control group, Cy control group, OBP low dose
(50mg/kg) group, OBP medium dose (100mg/kg) group, and OBP high dose (200mg/kg)
group. In the following 28 days, all mice had free access to tap water and food (ad libitum).
Besides, normal control group and Cy control group were given oral administration of
normal saline once a day, meanwhile the other three groups were given oral administration of
OBP by different dosage as 50mg/kg, 100mg/kg, 200mg/kg. At the day of 25 and 26, mice of
Cy control group and all OBP groups were submitted to Cy intraperitoneal injection
treatment (50mg/kg) once a day for 2 days to induce intestinal mucosal immunity
suppression, while the normal control group was submitted to normal saline i.p. injection as
control (Fig 1a). At the end of the feeding period, after overnight fasting, mice were
anaesthetized with diethyl ether. Blood was sampled from mice eyes, and then the animals
were sacrificed by cervical dislocation. Ileum was excised for further analysis. All aspects of
the experiment were conducted according to guidelines provided by the ethical committee of
experimental animal care at Ocean University of China (Qingdao, China).

2.4 RNA isolation and cDNA preparation

Total RNA was extracted from isolated ileum samples using Trizol reagent (Invitrogen,
USA). Each sample was dissolved in 0.5 ml Trizol reagent by homogenization in a
homogenizer, according to the manufacturer’s instructions. Protein and Trizol were removed
by addition of 0.1 ml chloroform. After isopropanol precipitation, centrifugation 12,000×g
for 10min, and washing by 75% ethanol, total RNA was extracted and dissolved in
diethylpyrocarbonate (DEPC) treated water. The amount and purity of RNA were quantified
spectrophotometrically by Nanodrop 2000c (Thermo Scientific, USA). RNA integrity was
checked by agarose gel electrophoresis. 1µg of RNA was converted to cDNA synthesis using
M-MLV reverse transcriptase (Promega, USA) and random primers (Sangon, China). The
cDNA samples were stored at -80°C until subsequent amplification for analysis.

2.5 Real-time quantitative polymerase chain reaction

Real-time PCR was performed in the Bio-Rad iCycler iQ5 system. 25 µL of reaction
volume was used for the quantitative real-time PCR assay that consisted of 12.5 µL Maxima
SYBR Green qPCR Master mix, 10 µM of primers (0.3 µL each of forward and reverse
primer), 5.9 µL nuclease-free water, and 6 µL of template. The thermal conditions consisted
of an initial denaturation at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C
for 15 s, annealing at 60 °C for 20 s and extension at 72 °C for 30 s. Data normalization was
accomplished using the endogenous reference β-actin and GAPDH (as they showed no
apparent difference in our previous studies28, 29, we used β-actin as endogenous reference
gene). The gene expression level was analyzed by relative quantification using the standard
curve method. The sequences of the primers used in this study are described in Table 1.

2.6 Mucins+ area evaluation on epithelium and quantity evaluation of goblet cells

The small intestine tissues were fixed in 4% phosphate buffered formalin (pH 7.0) for 24 h,
washed through running water, dehydrated through graded series of alcohols, cleaned in
methyl benzoate, and embedded in paraffin wax. Sections with a thickness of 5µm were
obtained, stained with Alcian blue-Periodic acid schiff (AB-PAS) staining method in
Qingdao Municipal Hospital (Department of pathology, Qingdao, China). Briefly, sections
were deparaffinized and hydrated to distilled water. Stained with Alcian blue (pH2.5) for 25
minutes, then washed in running tap water. After that, sections were oxidized in 1% periodic
acid (10 min), rinsed in distilled water, and treated with Schiff’s reagent (0.5% pararosaniline
wt/vol, 1% sodium metabisulfite wt/vol, 0.01 N HCl) for 15 min then dehydrated in ethanol
and xylene. Stained slides were coverslipped with antifade polyvinylpyrrolidone mounting
medium (Beyotime, China). Total mucin+ areas and quantities of goblet cell were measured
and counted using a digital image analysis system (Image pro plus software, Olympus
Optical Co. Ltd., Tokyo, Japan)\textsuperscript{30}. 10 images were taken from 5 slides for each group,
mucin+ areas were measured as pixels in every 5 microvilli (10 images) and goblet cell
quantities were counted as numbers in every 5 microvilli (10 images).

2.7 Transmission electron microscopy (TEM) scanning of ultrastructure of goblet cells

Small intestinal tissues were fixed at 4°C in 2.5% buffered glutaraldehyde for 1 h followed
by 1% osmium tetroxide for 2h. The tissues were dehydrated in ascending concentrations of
ethanol, immersed in propylene oxide, and embedded in Epon 812 resin (Agar Scientific Ltd.,
Standsted, England). The samples were cut in ultrathin sections (about 60 nm), contrasted
with 4% uranyl acetate and Reynold’s lead citrate, and examined in a Hitachi (H-7000)
electron microscope.

2.8 Statistical analysis
All the values in figures are expressed as mean ± standard error of the mean. Statistical comparisons of the results were performed using Tukey’s post-hoc test (ANOVA) analysis of variance by SPSS 11.0, $P<0.05$ was considered statistically significant.

3. Results

3.1 OBP ameliorated chemotherapy induced intestinal injury and protected goblet cells in Cy treated mice

Chemotherapy-induced diarrhea is a common side effect of cancer treatment and can cause significant morbidity and mortality. And constant diarrhea and other side effects cause weight loss and more pain to chemotherapy treated patients. In this study, we tested the bodyweight changes of all 5 group mice, by comparing the weight after chemotherapy treatment to the weight at the beginning of the feeding period. It showed that the bodyweight of all Cy treated mice decreased remarkably (Fig 1b). Nonetheless, comparing to the Cy control group mice, both 100mg/kg and 200mg/kg OBP administration ameliorated the bodyweight decrease significantly ($P<0.05$). And also, after the i.p. injection of Cy, there was diarrhea happened in all Cy treated mice. However, the diarrhea situation in 200mg/kg OBP mice was not as severe as that in Cy control group in our observation.

It is reported that mucus gel layers, by goblet cells secretion, play roles in protection against pathogen penetration and diarrhea caused by chemotherapy. Also, cytokeratin 18 (Cyto 18) and Mucin 2 (Muc 2) were highly expressed in goblet cells, as goblet cell markers. Thus, the relative expression levels of Cyto 18 and Muc 2 were detected in this study to evaluate the goblet cell function after chemotherapy and OBP treatment. Compared
to the normal control group, after i.p. treatment of Cy, the mRNA expression of Cyto 18 and Muc 2 decreased significantly ($P < 0.05$) in Cy group mice. However, OBP increased the mRNA expression levels of Cyto 18 and Muc 2, especially in the 200mg/kg OBP group ($P < 0.05$), compared to that in the Cy group (Fig 2 a, b).

### 3.2 Mucins expressed in intestinal goblet cells were up-regulated by OBP treatment in Cy treated mice

Furthermore, to confirm the protective effect of OBP on goblet cell secretion, the mucins contents which are secreted by goblet cells were studied by AB-PAS Staining. The mucins expression levels in 5 group mice were studied by calculating mucin$^+$ areas and mucin$^+$ cell numbers in AB-PAS stained intestinal sections (Fig 3). It showed that, mucins$^+$ area in intestinal villi was enhanced by OBP administration compared with that in Cy group (Fig 4 b), and it is in accordance with the previous Muc 2 mRNA expression result (Fig 2 b).

### 3.3 OBP regulated enhancement of Cyto 18 and Mucins expression in goblet cells relied on the relatively larger quantity of goblet cells

OBP triggered higher expression of Cyto 18 and mucins in goblet cells in the 200mg/kg OBP group than that in the Cy group. To gain further knowledge of which contribute to the enhancement of glycoproteins expression in goblet cells in OBP-treated mice, both the quantity and secretion capacity of goblet cell were investigated. It (Fig 3, Fig 4 c) implicated that Cy i.p. treatment remarkably decreased the quantity of goblet cell, compared with that in normal control group mice. Nonetheless, 200mg/kg OBP administration improved the
quantity of goblet cell in epithelium significantly ($P<0.01$) (Fig 4c). The ultrastructure of endoplasmic reticulum (ER) in goblet cells was further studied. Distinct with the quantity variance of goblet cells, there were no apparent differences of ER ultrastructure among these five group mice in goblet cells (Fig 5).

4. Discussion

The mucosal immune system is widely held to be responsible for the defense of the large expanse of mucous membranes that form a barrier between the external environment and the body’s interior. Goblet cell is one of the most characteristic cell types in epithelium for intrinsic mucosal immunity. This is particularly true for the intestinal tract. Various clinical and experimental studies have demonstrated that intestinal mucosal injury induced by chemotherapy impairs gut barrier function and leads to bacterial translocation, resulting in the systemic inflammatory response \(^{36, 37}\). Therefore, treatments that prevent intestinal mucosal injury following chemotherapy will comprise novel therapeutic strategies in maintaining gut barrier function and improving outcomes in patients receiving chemotherapy. This study aimed to enhance the intestinal mucosal innate immunity by oral administration of OBP. Besides, there is none report about functional food or compound to protect goblet cell from chemotherapy induced mucosal injury. Our study provides evidence that OBP is involved in stimulating intestinal mucosal innate immunity by promoting mucins secretion in goblet cells. The immunopotentiation effect of OBP on Cy i.p. treated mice was especially evident in the 200mg/kg OBP administrated mice.

Epithelial cells contribute to innate immunity by releasing antimicrobial proteins onto the
mucosal surfaces. They are covered by protective secretory mucins from the apical surface of goblet cells. In current study, Cy decreased the mucin glycoproteins secretion from goblet cells. It was demonstrated that mucin glycoproteins were increased significantly by oral administration of OBP. Ontogenic changes in the composition of intestinal mucus could correlate with successional changes in the inhabited microbiota and with regional maturation of acquired immune functions in intestinal homeostasis. There are studies about the effects of Cy on ER ultrastructural alterations of the cortical epithelial cells of the rat thymus and paneth cells of small intestine in mice, in which the cisternae of the ER was considerably dilated and vesiculated. The morphological change of ER would affect its secretory capacity. Thus, we further studied the effect of dietary OBP on ER ultrastructure of goblet cells. However, the ER structure of goblet cells was not changed by OBP feeding. It was confirmed that the enhancement of mucin secretion was due to OBP ameliorated the goblet cell quantity descending caused by Cy. In our previous studies, we also found the OBP could protect antimicrobial peptides secretion in paneth cells and IgA secretion in plasma cells. Hence further studies will be performed to confirm the present data and to investigate and compare the potential molecular mechanisms in OBP protecting goblet cells, paneth cells and plasma cells.

In conclusion, the present study suggests that functional OBP could reduce chemotherapeutic Cy induced small intestinal mucosal damage, by promoting mucin glycoproteins secretion by goblet cells. The enhancement of this intestinal mucosal innate immunity was dependent upon OBP stimulated quantity increase of goblet cells. This report indicates the utilizing potential of OBP in protecting against chemotherapy-induced mucosal damage.
injury.

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There is no conflict of interest
References:

Figure Legends

Fig 1. Dietary OBP ameliorated chemotherapy induced injury in mice. a) A schematic outline of the experimental timeline used for animal experiments. In the experimental 28 days, all 5 group mice had free access to tap water and food (ad libitum). Besides, mice of Normal control group and Cy control group were given oral administration of normal saline once a day, meanwhile the other three groups were given oral administration of OBP by different dosage as 50mg/(kg.bw), 100mg/(kg.bw), 200mg/(kg.bw). At the day of 25 and 26, mice of Cy control group and all OBP groups were submitted to Cy intraperitoneal injection treatment (50mg/(kg.bw)) once a day for 2 days to induce intestinal mucosal immunity suppressed, while the normal control group was submitted to normal saline i.p. injection as control. b) Dietary OBP ameliorated chemotherapy induced bodyweight loss in Cy treated mice. The bodyweight changes of all 5 group mice were calculated by comparing the weight after chemotherapy treatment to the weight at the beginning of the feeding period. Values are expressed as mean ± SEM. * P < 0.05, different from the Cy group.
Fig 2. OBP administration promoted the mRNA expression of Cyto 18 and Muc 2 in goblet cells. Relative mRNA expression of Cyto 18 (a), Muc 2 (b) were studied by RT-qPCR. Values are expressed as mean ± SEM. # P < 0.05, different from the Normal group; * P < 0.05, **P < 0.01 different from the Cy group.

Fig 3. AB-PAS Staining of intestinal sections. AB-PAS Staining of small intestine sections in 5 group mice (a) and their zoomed-in pictures (b), arrows indicated the mucin glycoprotein in goblet cell.
Fig 4. OBP administration promoted the expression of mucins in goblet cells and enhanced the quantity of goblet cells in small intestine. (a) Sample of AB-PAS Stained small intestine sections, arrows indicated the mucin glycoproteins in goblet cells, scal bar indicates 25µm. (b) The calculated grayscale of mucins in Fig 3b. (c) Statistical analysis of the relative quantities of goblet cells in small intestine by counting goblet cells in small intestine as indicated in Fig 3b. Values are expressed as mean ± SEM. # P < 0.05, different from the Normal group; * P < 0.05, **P < 0.01 different from the Cy group.

Fig 5. OBP showed no apparent effect on the ultrastructure of ER in goblet cells. TEM Scanning of the ultrastructure of ER in goblet cells of 5 groups mice, scal bars indicate 0.5 µm. Arrows indicate the ER in the goblet cells.
Table 1 Primers used in this study

<table>
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<th>gene</th>
<th>forward primer (5'-3')</th>
<th>reverse primer (5'-3')</th>
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<td>TGCTGATCCACATCTGCTGG</td>
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