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

3

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14 Abstract: Gastrointestinal mucositis induced by chemotherapy is associated with alterations
15 of intestinal barrier function due to the potential damage induced by anti-cancer drugs on the
16 epithelial cells. Goblet cell, an important epithelial lineage in the intestine, contributes to
17 innate immunity by secreting mucin glycoproteins. Employing a mouse model of
18 chemotherapy induced intestinal mucosal immunity injury by cyclophosphamide, we
19 demonstrated for the first time that polysaccharide from the ink of *Ommastrephes bartrami*
20 (OBP) enhanced Cyto18, mucin expression in goblet cells. The up-regulation of mucins by
21 OBP relied on the augmented quantity of goblet cells, but not on the changes in endoplasmic
22 reticulum (ER) ultrastructure. Our results may have important implications for enhanced
23 immunopotentiation function of functional OBP on intestinal mucosal immunity against
24 intestinal disorders involving inflammation and infection.

25

26 Key words: squid ink, polysaccharide, goblet cells, mucin

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28 **1. Introduction**

29 Mucosal barriers are endowed with multi-functional defense mechanisms that selectively
30 handle harmful or innocuous antigens to ensure local homeostasis. The gastrointestinal tract,
31 which is part of the mucosal system, is exposed to a multitude of ingested or inhaled
32 microorganisms, environmental and food antigens. In particular, the vulnerable intestinal
33 mucosa is persistently exposed to potentially harmful ingested agents. Various clinical
34 studies have shown that chemotherapy could damage host immunity system, and cause
35 intestinal microflora imbalance [1]. Infection is one of the most commonly encountered
36 complications during chemotherapy treatment, resulting in gastrointestinal disorders, such as
37 persistent diarrhea, stomachache, emesis, and bacterial systemic dissemination [2-4]. That is
38 extremely painful for chemotherapeutic patients, which make it urgent and imperative to find
39 a cure to reduce their pains. Because of the side-effects of pharmaceutical products,
40 functional food or its natural bioactive components become a relatively good choice for
41 chemotherapeutic patients.

42 The goblet cell, which is one of four major epithelial cell lineages in the small intestine, is
43 part of the first-line protection of the mucosal surface that belongs to the innate mucosal
44 immune system for the host's defense against possible pathogens. Gastrointestinal epithelium
45 is covered with protective mucus composed predominantly of mucin glycoproteins which are
46 synthesized and secreted by goblet cells. The mucin glycoproteins act as a medium for
47 lubrication, protection between the luminal contents and the epithelial lining, and more
48 importantly, preventing gut bacteria penetrating the epithelium barrier [5]. Recent evidence
49 revealed that goblet cells deliver luminal antigen to $CD103^+$ dendritic cells in the small

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50 intestine [6], which gives us another understanding of how pathogen-specific immunity is
51 elicited while avoiding inappropriate responses to the background of innocuous antigens.
52 The proper functions of goblet cells are essential for treating against intestinal infections and
53 inflammatory diseases.

54 Chemotherapeutic cyclophosphamide (Cy) is an anti-tumor drug with a wide spectrum of
55 clinical uses and it has been proved to be effective in the treatment of cancer and
56 nonmalignant disease states such as rheumatoid arthritis [7-10]. However, high doses of
57 anti-cancer drugs can damage the intestinal mucosa which can lead to clinical problems such
58 as bacterial translocation, diarrhea and dyskinesia [11-13]. The complications of anti-cancer
59 chemotherapy include gastrointestinal (GI) mucositis, which represents injury of the rest of
60 the alimentary tract beyond oral mucositis [14]. This condition is most prominent in the small
61 intestine, but it also occurs in the esophagus, stomach, and large intestine [15]. GI mucositis
62 induced by chemotherapy is associated with alterations of intestinal barrier function [16] due to
63 the potential damage induced by the anti-cancer drugs on the epithelial cells of the intestinal
64 mucosa. Cytotoxic drugs impair the turn-over of intestinal epithelia, induce flattening of the
65 villi and increase the exposure of luminal contents to crypts [17]. In this process, goblet cells
66 which belong to intestinal epithelia, will be under attack by cyclophosphamide.

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

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72 immunity.

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

91

92 **2. Materials and Methods**

93 *2.1 Materials*

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94 The ink sac of the squid, *Ommastrephes bartrami*, was obtained from Zhou-Shan
95 Fishery Company (Zhejiang, China) and stored at −20 °C before use. TRIzol reagent was
96 obtained from Invitrogen (Carlsbad, USA). M-MLV reverse transcriptase was obtained from
97 Promega (Madison, WI). Maxima SYBR Green qPCR Master mix was purchased from
98 Fermentas (Glen Burnie, Maryland). Cyclophosphamide was purchased from Jiangsu
99 Hengrui Medicine Co., Ltd (Jiangsu, China).
100 All the chemical reagents used in the experiment were of analytically purity.
101
102 *2.2 Preparation of OBP*
103 OBP was prepared as previously reported by *Chen et al* [27]. Briefly, after squid ink of
104 *Ommastrephes bartrami* was acidified to pH 4–5 with 0.1 M HCl and the solution stood for
105 24 h at 4°C to precipitate melanin, melanin was removed by centrifugation at 5,000×g for 1
106 h. Then melanin-free ink was digested with 2 volumes of 1% (w/v) papain in Tris–HCl
107 buffer (50mM, pH 6.8) containing 5mM Cys and 5mM EDTA at 60°C for 24 h. Digestion
108 was repeated twice to ensure the cleavage of the protein/peptide moiety. Melanin-free
109 polysaccharide was obtained after precipitation with 4 volumes of ethanol. The resultant
110 OBP extract was dialyzed against several changes of water and then lyophilized.
111
112 *2.3 Animals*
113 Male Balb/c mice weighting 18 to 22 g were purchased from Vital River Laboratory
114 Animal Center (Beijing, China). The mice were housed throughout the feeding experiment in
115 a room maintained at a 12 hours light/dark cycle, a constant temperature of 24°C, and a

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116 relative humidity of 65±15%. After a 7 days adaptation period, mice were assigned to five
117 groups with 10 mice each group: Normal control group, Cy control group, OBP low dose
118 (50mg/kg) group, OBP medium dose (100mg/kg) group, and OBP high dose (200mg/kg)
119 group. In the following 28 days, all mice had free access to tap water and food (ad libitum).
120 Besides, normal control group and Cy control group were given oral administration of
121 normal saline once a day, meanwhile the other three groups were given oral administration of
122 OBP by different dosage as 50mg/kg, 100mg/kg, 200mg/kg. At the day of 25 and 26, mice of
123 Cy control group and all OBP groups were submitted to Cy intraperitoneal injection
124 treatment (50mg/kg) once a day for 2 days to induce intestinal mucosal immunity
125 suppression, while the normal control group was submitted to normal saline i.p. injection as
126 control (Fig 1a). At the end of the feeding period, after overnight fasting, mice were
127 anaesthetized with diethyl ether. Blood was sampled from mice eyes, and then the animals
128 were sacrificed by cervical dislocation. Ileum was excised for further analysis. All aspects of
129 the experiment were conducted according to guidelines provided by the ethical committee of
130 experimental animal care at Ocean University of China (Qingdao, China).
131

132 *2.4 RNA isolation and cDNA preparation*

133 Total RNA was extracted from isolated ileum samples using Trizol reagent (Invitrogen,
134 USA). Each sample was dissolved in 0.5 ml Trizol reagent by homogenization in a
135 homogenizer, according to the manufacturer's instructions. Protein and Trizol were removed
136 by addition of 0.1 ml chloroform. After isopropanol precipitation, centrifugation 12,000×g
137 for 10min, and washing by 75% ethanol, total RNA was extracted and dissolved in

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138 diethylpyrocarbonate (DEPC) treated water. The amount and purity of RNA were quantified
139 spectrophotometrically by *Nanodrop 2000c* (Thermo Scientific, USA). RNA integrity was
140 checked by agarose gel electrophoresis. 1μg of RNA was converted to cDNA synthesis using
141 M-MLV reverse transcriptase (Promega, USA) and random primers (Sangon, China). The
142 cDNA samples were stored at -80°C until subsequent amplification for analysis.

143

144 *2.5 Real-time quantitative polymerase chain reaction*

145 Real-time PCR was performed in the Bio-Rad iCycler iQ5 system. 25 μL of reaction
146 volume was used for the quantitative real-time PCR assay that consisted of 12.5 μL Maxima
147 SYBR Green qPCR Master mix, 10 μM of primers (0.3 μL each of forward and reverse
148 primer), 5.9 μL nuclease-free water, and 6 μL of template. The thermal conditions consisted
149 of an initial denaturation at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C
150 for 15 s, annealing at 60 °C for 20 s and extension at 72 °C for 30 s. Data normalization was
151 accomplished using the endogenous reference *β*-actin and GAPDH (as they showed no
152 apparent difference in our previous studies[28, 29], we used *β*-actin as endogenous reference
153 gene). The gene expression level was analyzed by relative quantification using the standard
154 curve method. The sequences of the primers used in this study are described in **Table 1**.

155

156 *2.6 Mucins$^+$ area evaluation on epithelium and quantity evaluation of goblet cells*

157 The small intestine tissues were fixed in 4% phosphate buffered formalin (pH 7.0) for 24 h,
158 washed through running water, dehydrated through graded series of alcohols, cleaned in
159 methyl benzoate, and embedded in paraffin wax. Sections with a thickness of 5μm were

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160 obtained, stained with Alcian blue-Periodic acid schiff (AB-PAS) staining method in
161 Qingdao Municipal Hospital (Department of pathology, Qingdao, China). Briefly, sections
162 were deparaffinized and hydrated to distilled water. Stained with Alcian blue (pH2.5) for 25
163 minutes, then washed in running tap water. After that, sections were oxidized in 1% periodic
164 acid (10 min), rinsed in distilled water, and treated with Schiff's reagent (0.5% pararosaniline
165 wt/vol,1%sodium metabisulfite wt/vol, 0.01 N HCl) for 15 min then dehydrated in ethanol
166 and xylene. Stained slides were coverslipped with antifade polyvinylpyrrolidone mounting
167 medium (Beyotime, China). Total mucin^{+} areas and quantities of goblet cell were measured
168 and counted using a digital image analysis system (Image pro plus software, Olympus
169 Optical Co. Ltd., Tokyo, Japan) [30]. 10 images were taken from 5 slides for each group,
170 mucin^{+} areas were measured as pixels in every 5 microvilli (10 images) and goblet cell
171 quantities were counted as numbers in every 5 microvilli (10 images).
172
173 *2.7 Transmission electron microscopy (TEM) scanning of ultrastructure of goblet cells*
174 Small intestinal tissues were fixed at 4°C in 2.5% buffered glutaraldehyde for 1 h followed
175 by 1% osmium tetroxide for 2h. The tissues were dehydrated in ascending concentrations of
176 ethanol, immersed in propylene oxide, and embedded in Epon 812 resin (Agar Scientific Ltd.,
177 Standsted, England). The samples were cut in ultrathin sections (about 60 nm), contrasted
178 with 4% uranyl acetate and Reynold's lead citrate, and examined in a Hitachi (H-7000)
179 electron microscope.
180
181 *2.8 Statistical analysis*

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182 All the values in figures are expressed as mean ± standard error of the mean. Statistical
183 comparisons of the results were performed using Tukey's post-hoc test (ANOVA) analysis of
184 variance by *SPSS 11.0*, $P<0.05$ was considered statistically significant.
185

186 **3. Results**

187 *3.1 OBP ameliorated chemotherapy induced intestinal injury and protected goblet cells in*
188 *Cy treated mice*

189 Chemotherapy-induced diarrhea is a common side effect of cancer treatment and can
190 cause significant morbidity and mortality [31]. And constant diarrhea and other side effects
191 cause weight loss and more pain to chemotherapy treated patients. In this study, we tested the
192 bodyweight changes of all 5 group mice, by comparing the weight after chemotherapy
193 treatment to the weight at the beginning of the feeding period. It showed that the bodyweight
194 of all Cy treated mice decreased remarkably (Fig 1b). Nonetheless, comparing to the Cy
195 control group mice, both 100mg/kg and 200mg/kg OBP administration ameliorated the
196 bodyweight decrease significantly ($P < 0.05$). And also, after the i.p. injection of Cy, there
197 was diarrhea happened in all Cy treated mice. However, the diarrhea situation in 200mg/kg
198 OBP mice was not as severe as that in Cy control group in our observation.

199 It is reported that mucus gel layers, by goblet cells secretion, play roles in protection
200 against pathogen penetration and diarrhea casused by chemotherapy [32-34]. Also, cytokeratin
201 18 (Cyto 18) and Mucin 2 (Muc 2) were highly expressed in goblet cells, as goblet cell
202 markers [6, 35]. Thus, the relative expression levels of Cyto 18 and Muc 2 were detected in this
203 study to evaluate the goblet cell function after chemotherapy and OBP treatment. Compared

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204 to the normal control group, after i.p. treatment of Cy, the mRNA expression of Cyto 18 and
205 Muc 2 decreased significantly ($P < 0.05$) in Cy group mice. However, OBP increased the
206 mRNA expression levels of Cyto 18 and Muc 2, especially in the 200mg/kg OBP group ($P <
207 0.05$), compared to that in the Cy group (Fig 2 a, b).

208

209 *3.2 Mucins expressed in intestinal goblet cells were up-regulated by OBP treatment in Cy*
210 *treated mice*

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

217

218 *3.3 OBP regulated enhancement of Cyto 18 and Mucins expression in goblet cells relied on*
219 *the relatively larger quantity of goblet cells*

220 OBP triggered higher expression of Cyto 18 and mucins in goblet cells in the 200mg/kg
221 OBP group than that in the Cy group. To gain further knowledge of which contribute to the
222 enhancement of glycoproteins expression in goblet cells in OBP-treated mice, both the
223 quantity and secretion capacity of goblet cell were investigated. It (Fig 3, Fig 4 c) implicated
224 that Cy i.p. treatment remarkably decreased the quantity of goblet cell, compared with that in
225 normal control group mice. Nonetheless, 200mg/kg OBP administration improved the

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226 quantity of goblet cell in epithelium significantly (*P<0.01*) (Fig 4 c). The ultrastructure of
227 endoplasmic reticulum (ER) in goblet cells was further studied. Distinct with the quantity
228 variance of goblet cells, there were no apparent differences of ER ultrastructure among these
229 five group mice in goblet cells (Fig 5).
230

231 **4. Discussion**

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

247 Epithelial cells contribute to innate immunity by releasing antimicrobial proteins onto the

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248 mucosal surfaces. They are covered by protective secretory mucins from the apical surface of
249 goblet cells. In current study, Cy decreased the mucin glycoproteins secretion from goblet
250 cells. It was demonstrated that mucin glycoproteins were increased significantly by oral
251 administration of OBP. Ontogenic changes in the composition of intestinal mucus could
252 correlate with successional changes in the inhabited microbiota and with regional maturation
253 of acquired immune functions in intestinal homeostasis [38]. There are studies about the effects
254 of Cy on ER ultrastructural alterations of the cortical epithelial cells of the rat thymus and
255 paneth cells of small intestine in mice, in which the cisternae of the ER was considerably
256 dilated and vesiculated [28, 39]. The morphological change of ER would affect its secretory
257 capacity. Thus, we further studied the effect of dietary OBP on ER ultrastructure of goblet
258 cells. However, the ER structure of goblet cells was not changed by OBP feeding. It was
259 confirmed that the enhancement of mucin secretion was due to OBP ameliorated the goblet
260 cell quantity descending caused by Cy. In our previous studies, we also found the OBP could
261 protect antimicrobial peptides secretion in paneth cells and IgA secretion in plasma cells [26, 28].
262 Hence further studies will be performed to confirm the present data and to investigate and
263 compare the potential molecular mechanisms in OBP protecting goblet cells, paneth cells and
264 plasma cells.

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

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270 injury.

271

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277

278 **There is no conflict of interest**

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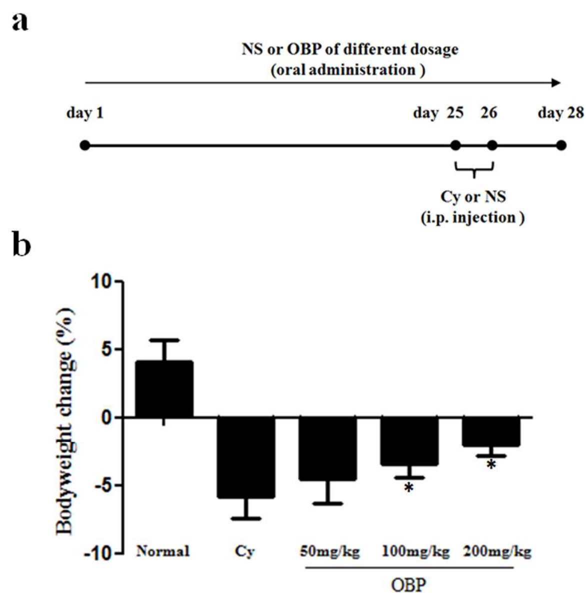
344 **Figure Legends**



345

346 **Fig 1. Dietary OBP ameliorated chemotherapy induced injury in mice.** a) A schematic
347 outline of the experimental timeline used for animal experiments. In the experimental 28
348 days, all 5 group mice had free access to tap water and food (ad libitum). Besides, mice of
349 Normal control group and Cy control group were given oral administration of normal saline
350 once a day, meanwhile the other three groups were given oral administration of OBP by
351 different dosage as 50mg/(kg.bw), 100mg/(kg.bw), 200mg/(kg.bw). At the day of 25 and 26,
352 mice of Cy control group and all OBP groups were submitted to Cy intraperitoneal injection
353 treatment (50mg/(kg.bw)) once a day for 2 days to induce intestinal mucosal immunity
354 suppressed, while the normal control group was submitted to normal saline i.p. injection as
355 control. b) Dietary OBP ameliorated chemotherapy induced bodyweight loss in Cy treated
356 mice. The bodyweight changes of all 5 group mice were calculated by comparing the weight
357 after chemotherapy treatment to the weight at the beginning of the feeding period. Values are
358 expressed as mean ± SEM. * $P < 0.05$, different from the Cy group.

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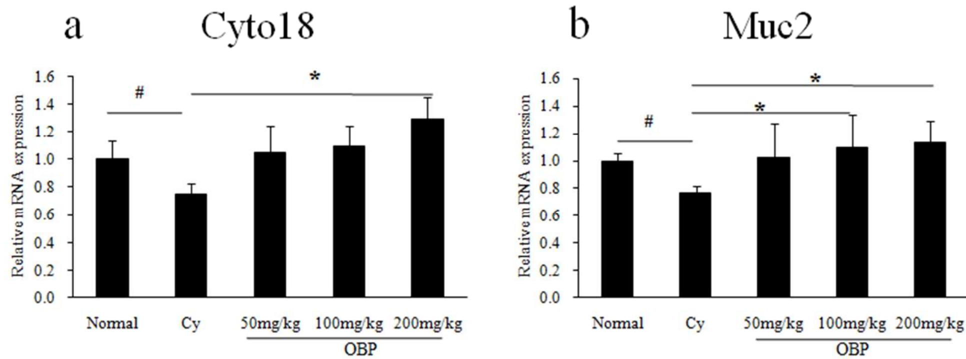


359

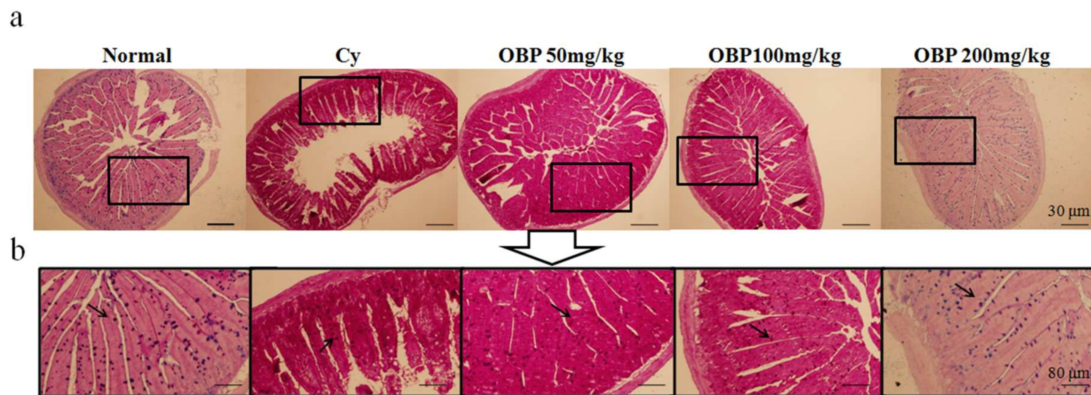
360 **Fig 2. OBP administration promoted the mRNA expression of Cyto 18 and Muc 2 in**

361 **goblet cells.** Relative mRNA expression of Cyto 18 (a), Muc 2 (b) were studied by RT-qPCR.

362 Values are expressed as mean ± SEM. # $P < 0.05$, different from the Normal group; * $P <$

363 0.05, **$P < 0.01$ different from the Cy group.

364



365

366 **Fig 3. AB-PAS Staining of intestinal sections.** AB-PAS Staining of small intestine sections

367 in 5 group mice (a) and their zoomed-in pictures (b), arrows indicated the mucin

368 glycoprotein in goblet cell.

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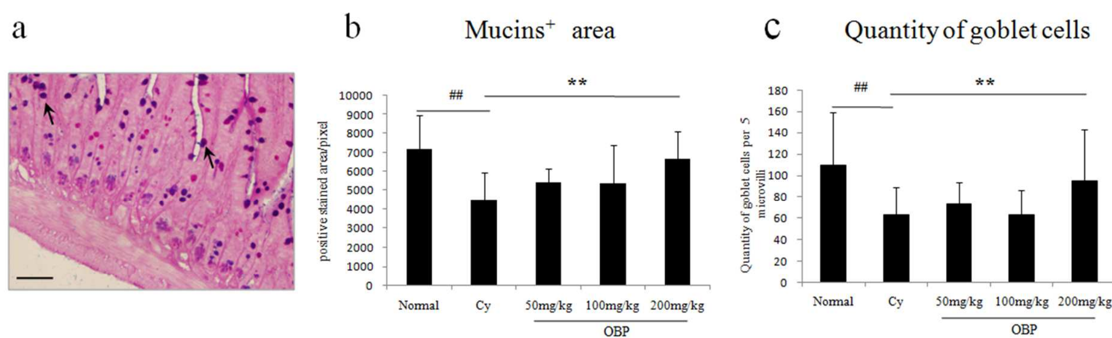
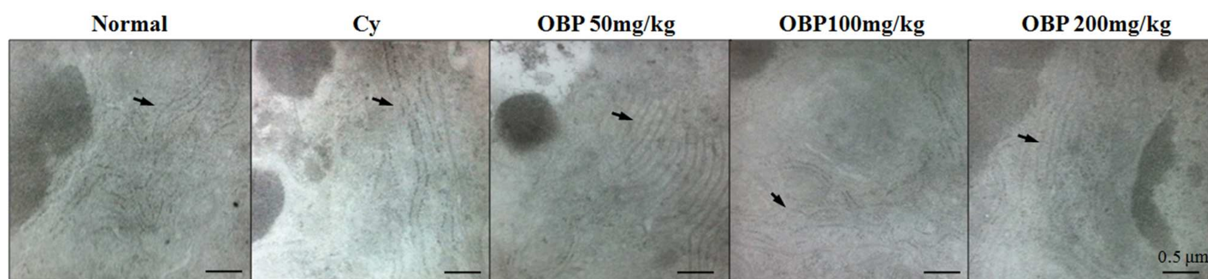


369

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



377

378 **Fig 5. OBP showed no apparent effect on the ultrastructure of ER in goblet cells.**
379 TEM Scanning of the ultrastructure of ER in goblet cells of 5 groups mice, scal bars indicate
380 0.5 μm. Arrows indicate the ER in the goblet cells.
381

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382 Table 1 Primers used in this study

gene	forward primer(5'-3')	reverse primer(5'-3')
β-actin	CAGGCATTGCTGACAGGATG	TGCTGATCCACATCTGCTGG
Cyto18	CAGCCAGCGTCTATGCAGG	CTTTCTCGGTCTGGATTCCAC
Muc2	CACACAGCGGCCTTTCTCAT	ACCCTCCTCCTACCACATTG

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