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Chongkukjang Mucilage Stimulates Immunohistochemical Activities of Gastrointestinal Tract in Rats

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Abstract We investigated the effect of a viscous substance from *chongkukjang* (*chongkukjang* mucilage) on immunohistochemical reactions in rat gastrointestinal (GI) tracts. Rats fed a steady diet of *chongkukjang* mucilage showed an increase in the immunoreactive densities of gastrin and serotonin in the pyloric region of their stomachs and duodenal villi. The number of gastrin and serotonin immunoreactive cells was significantly higher in the experimental group than in the control group. Feeding on dietary *chongkukjang* mucilage increased the immunohistochemical densities of $CD4^+$ and $CD8^+$ lymphocytes in the mucosa and submucosa of the rats' gastroduodenal region. The universal nitric oxide synthase (uNOS)-immunoreactive neurons and nerve fibers were strongly stained in the vascular walls of the submucosa and myenteric plexus in rats fed the test diet. The results indicate that the intake of *chongkukjang* mucilage could increase mucosal immune activity, gastrointestinal motility, and blood circulation in the GI tract.

Keywords: *Chongkukjang* mucilage, gastrin and serotonin immunoreactive cells, $CD4^+$ and $CD8^+$ lymphocytes, uNOS immunoreactive cells

Introduction

Chongkukjang, a soybean- based traditional fermented food, has been eaten for several hundreds of years in Korea. It is prepared by fermenting steamed whole unsalted soybeans with *Bacillus subtilis*, the major species. This product is a good source of essential amino acids and fatty acids in the Korean diet, which suggests its potential as both a nutritional and healthy food (1). It is also well known that *Chongkukjang* possesses various physiological properties (biological activities) in serum: fibrinolytic activity, anticancer activity, antihypertensive activity, as well as hypocholesterolemic and hypolipidemic effects (2-6). These physiological properties could be attributed to a viscous substance produced by the actions of various enzymes from microbes of the genus *Bacillus* during fermentation.

The *chongkukjang* mucilage is composed of fructan in the form of levan and polyglutamate, and provides a unique taste and flavor to the product (7). A few studies have reported on the production and physicochemical properties of this substance (8, 9). However, little information is available on how *Chongkukjang* mucilage affects physiological properties.

In this study, we examined the effects of the *chongkukjang* mucilage on immunohistochemical reactions in the gasrointestinal (GI) tract of rats. After the administration of *chongkukjang* mucilage, the immunohistochemical densities of enteroendocrine cells (gastrin and serotonin), immunocompetent cells ($CD4^+$ and $CD8^+$), and universal nitric oxide synthase (uNOS) in the GI tract were observed using a process of immunohistochemical staining. We also investigated whether the intake of viscous substance modulated the intestinal motility and mucosal immune system in the GI tract.

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Materials and Methods

Separation of viscous substance from *chongkukjang* mucilage The *chongkukjang* mucilage was separated from *chongkukjang* using the following method. *Chongkukjang* (300 g) purchased from Sunchang County, Korea was shaken for 30 min (20°C, 220 rpm) after adding distilled water (2 L). It was then filtered with cheese-clothe to separate the solid material from the viscous substance. The filtrate (viscous substance fraction) was centrifuged at 10,000 × g for 20 min, and the supernatant was freeze dried. The *chongkukjang* mucilage was composed of 61.3% crude protein, 25.3% carbohydrate, 0.1% lipid and 13.3% crude ash.

Animals and diets Male Sprague-Dawley rats (6 weeks old, Samtako, Korea) were randomly assigned to two groups. The composition of the experimental diet is presented in Table 1. The experimental group was administered a diet that contained 6% *chongkukjang* mucilage for 4 weeks. The control rats were fed a basal diet. The rats were supplied with water and feed *ad libitum*.

Tissue processing Six rats were sacrificed after experimental diets were provided for 4 weeks. The pyloric portion of the stomach and proximal duodenum were excised, placed in 10% neutral buffered formalin, and prepared for routine paraffin embedding. Sections of tissue were cut into 7 μm slices using a microtome and mounted

Table 1. Composition of experimental diets (g/100g diet)

Ingredients	Dietary group	
	Control	*Chongkukjang* mucilage
Casein	20	20
DL-Methionine	0.3	0.3
Sucrose	55	55
Corn starch	10	10
Corn oil	5	5
Mineral mixture[a]	3.5	3.5
Vitamin mixture[b]	1	1
Choline bitartate	0.2	0.2
Cellulose	5	5
Chongkukjang mucilage	-	6

[a,b]AIN-76

onto slides. Tissue sections were stained using immunohistochemical methods.

Immunohistochemical staining Immunohistochemical staining was measured as described by Hsu *et al.* (10). Tissue sections (GI tract) were deparaffinized with xylene and hydrated with a graded alcohol series. Because certain antigenic determinants were masked by formalin fixation, tissue slides were placed in a container and covered with a 10 mM sodium citrate buffer heated at 95°C for 5 min. Tissue slides were allowed to cool in the buffer for 20 min. These specimens were then washed in distilled water three times for 2 minutes each. To prevent potential endogenous peroxidase activity, tissue sections were incubated for 10 min in PBS containing 1% H_2O_2. Next, the tissue sections were incubated for 1 h in PBS containing 5% normal goat serum. Certain sections were then transferred to rabbit anti-gastrin (diluted 1:300, Dako, Carpinteria, CA, USA), rabbit anti-serotonin (diluted 1:1000, Sigma, St. Louise, MO, USA), rat anti-CD4 (diluted 1:50, Santa CruzBiotechnology, Santa Cruz, CA, USA), rat anti-CD8 (diluted 1:50, Santa CruzBiotechnology, Santa Cruz, CA, USA) and rabbit anti-uNOS (diluted 1:50, Sigma, St. Louise, MO, USA) in PBS containing 0.3% Triton X-100 and 2% bovine serum albumin. Samples were incubated overnight at 4°C. After washing the specimens two times for 10 min in PBS, sections were incubated sequentially in biotinylated anti-rabbit IgG and biotinylated anti-rat IgG (Vector, USA). The sections were then diluted 1:200 in the same solution as the primary antiserum for 2 h. These specimens were incubated with an avidin-biotin enzyme for 1 h, and then washed again in PBS. The sections were visualized with 3,3'-diaminobenzidine(DAB) in a 0.1 M Tris buffer and mounted on gelatin-coated slides. Any immunoreactions were observed under an Axioscope microscope (Carl Zeiss, Germany).

Count of immunoreactive cells Immunoreactive cells were counted using a magnification of 40× objective lens and 10× ocular lens. The optical visual field was 0.96 mm, and 10 slides were counted per sample.

Statistical analysis Data was analyzed by ANOVA using the SAS statistical analysis system (SAS Institute Inc., Cary, NC, USA). Differences among samples were analyzed using Duncan's multiple range test ($P < 0.05$).

Results and Discussion

Gastrin and serotonin immunoreactive cells Gastrin is a well-known substance for stimulating the secretion of gastric acid and three types of pepsinogens, as well as the growth of gastric mucosa. It is also associated with a mechanism of gastrointestinal motility involving the cholinergic nervous system (11). Serotonin, which is released by enterochromaffin cells in the mucosal epithelium (12), stimulates the secretion of duodenal mucosal bicarbonate and modulates many gastrointestinal functions (13).

Table 2 shows the results for immunoreactive cells of gastrin and serotonin in gastrointestinal tracts after administration of *chongkukjang* mucilage for 4 weeks. The number of gastrin and serotonin immunoreactive cells in the pyloric region of the stomach was significantly higher in the *chongkukjang* mucilage diet group than in the basal diet group. Immunohistochemical density in the basal region of mucosa appeared strong in the group administered the *chongkukjang* mucilage. The number of gastrin and serotonin immunoreactive cells in the proximal region of the duodenum was also significantly higher in rats on a *chongkukjang* mucilage diet. Immunohistochemical density in lamina propria of villus was expressed mildly in rats fed the basal (control) diet, and was expressed strongly and distributed in the villus tip of rats that consumed *chongkukjang* mucilage. A significant increase in the number of gastrin immunoreactive cells and a strong expression of immunohistochemical density indicated that gastrointestinal motility could be increased by intake of *chongkukjang* mucilage (Fig. 1). The significantly elevated number of serotonin immunoreactive cells and the strong expression of immunohistochemical density (Fig. 2) also indicated that both pancreatic secretions, and small bowel motility could be increased with intake of the *chongkukjang* mucilage (14). According to Ovsiannikov and Berezina (15), small bowel motility is

Table 2. Numerical changes of gastrin and serotonin in gastrointestinal tract after administration of *chongkukjang* mucilage to rats for 4 weeks

	Gastrin		Serotonin	
	Stomach	Duodenum	Stomach	Duodenum
Control	26.4±5.6[b]	1.7±1.6[b]	15.7±4.5[b]	9.3±2.5[b]
Chongkukjang mucilage	40.9±9.6[a]	11.7±4.0[a]	47.0±10.0[a]	12.3±2.7[a]

Values are expressed as a mean±SD for six rats.
Values followed by different letters within columns are significantly different at $\alpha=0.05$ by Duncan's multiple test.

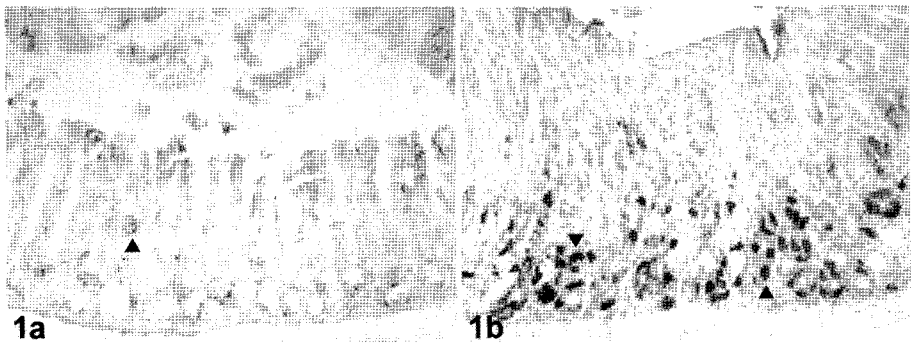

Fig. 1. Photomicrographs of stained gastrin immunoreactive cells in gastric mucosa of the control (1a) and *chongkukjang* mucilage (1b) diet groups (×100). Arrow heads represent weakly and heavily stained gastrin-secreting cells.

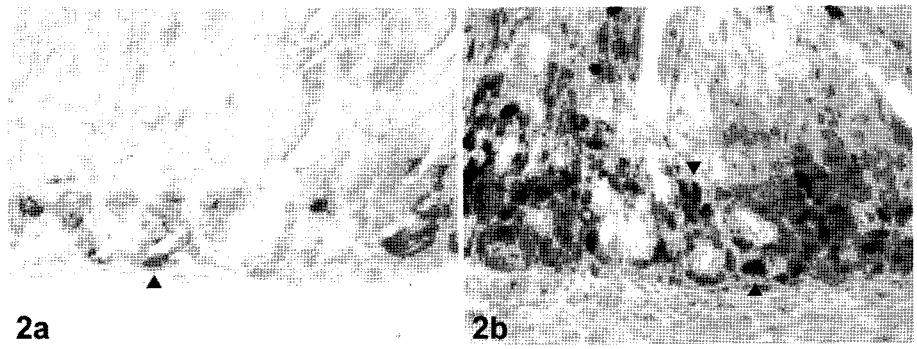

Fig. 2. Photomicrographs of stained serotonin immunoreactive cells in gastric mucosa of control (2a) and *chongkukjang* mucilage (2b) dietgroups (×200). Arrow heads represent mildly and heavily stained serotonin- secreting cells.

stimulated by serotonin's activation of the non-cholinergic excitatory mechanism with the participation of a nonadrenergic noncholinergic inhibitory mechanism.

$CD4^+$ and $CD8^+$ immunoreactive cells Lymphoid organs, which contain lymphoid follicles, lamina propria lymphocytes, and intraepithelial lymphocytes, have been known to mediate various forms of cytotoxicity (16, 17) and secreted cytokines (18). They play important roles in immune responses to foreign antigens as well as in the prevention of diseases of the intestinal tract. Intraepithelial lymphocytes (IELs) play a particularly significant role in the maintenance of epithelial homeostasis, and highlight a significant relationship between short chain fatty acids (SCFA) and the localization of immune cells ($CD4^+$ IELs and $CD8^+$ IELs) (19).

Table 3 shows the immunohistochemical density of $CD4^+/CD8^+$ lymphocytes in gastrointestinal tracts after the administration of *chongkukjang* mucilage for 4 weeks. In the stomachs of control group rats, $CD4^+$ immunoreactive cells in the lamina propria of mucosal base were weakly stained. The stomachs of the control rats also exhibited mildly stained cells in their submucosal perivascular and intravasular areas. The experimental diet group experienced increased immunohistochemical densities of $CD4^+/CD8^+$ in the secretory epithelium and lamina propria of the mucosa, as well as the submucosa, of their stomachs.

In the duodenum cells of the control group, there were weakly stained $CD4^+$ immunoreactive cells in the lamina

Table 3. Immunohistochemical density of $CD4^+/CD8^+$ lymphocytes in gastrointestinal tract after administration of *chongkukjang* mucilage to rats for 4 weeks

		Mucosa		Submucosa	
		$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
Stomach	Control	+	+	++	+
	Chongkukjang mucilage	+++	+++	+++	+++
Duodenum	Control	+	+	+++	+++
	Chongkukjang mucilage	+++	+++	+++	+++
Jejunum	Control	+	+	+	+
	Chongkukjang mucilage	++	++	++	++
Colon	Control	+	+	+	+
	Chongkukjang mucilage	+	+	++	+++

Immunoreactive density: + weak, ++ moderate, +++ strong

propria and intraepithelial cells of mucosal villi, while there were strongly stained cells in the submucosa. This group also had weakly stained $CD8^+$ immunoreactive cells at the base of the mucosa and strongly stained cells in the submucosa. The *chongkukjang* mucilage diet group showed increased immunohistochemical densities of $CD4^+/CD8^+$ in mucosal lamina propria, and in the perivascular area of the submucosa.

In the jejunum, the control group exhibited weakly stained $CD4^+/CD8^+$ immunoreactive cells in the mucosal and submucosal lamina propria. In contrast, the *chongkukjang* mucilage diet group exhibited increased immunohistochemical densities of $CD4^+/CD8^+$ in lamina propria of the mucosa and submucosa.

In the colon region, the control diet group presented weak $CD4^+/CD8^+$ immunoreactive densities in the mucosal and submucosal lamina propria. The experimental diet group exhibited weakly stained $CD4^+/CD8^+$ immunohistochemical densities in the mucosal lamina propria. It also showed strongly stained $CD8^+$ immunoreactive cells in the submucosa.

Some dietary fibers have been observed to contribute to an increase in the densities of $CD8^+$ IELs and $CD4^+$ IELs in the colon. Ingestion of some dietary fiber modulates local cell proliferation of $CD8^+$ IEL or promotes homing of $CD8^+$ T cells into the large intestinal epithelium through fermentation in the luminal contents (19). A significant increase in immunoreactive $CD4^+/CD8^+$ cells in mucosa and submucosa of the stomach and duodenum by administering a *chongkukjang* mucilage diet could modulate immune response in the GI tract.

Universal nitric oxide synthase (uNOS) immunoreactive cells and fibers Table 4 demonstrates that uNOS was widely distributed in the GI tract. Most of them were located in the myenteric plexus and distributed in the submucosal plexus, mucosal epithelium, and gland. The control group exhibited weakly stained uNOS immunoreactive cells in their GI tracts. However, the *chongkukjang* mucilage diet group had strongly stained uNOS immunoreactive cells in the myenteric plexus of their stomachs' muscle layer. The *chongkukjang* mucilage diet group presented strongly stained uNOS immunoreactive cells on surface epithelium of the mucosa, and mildly stained cells in the myenteric plexus of the muscle layer of the duodenum. In the jejunum, the *chongkukjang* mucilage diet group had weakly stained uNOS immunoreactive cells in surface epthelium of the mucosa and submucosa. In the colon region, the *chongkukjang* mucilage diet group exhibited mildly stained uNOS immunoreactive cells on surface epthelium of the mucosa and myenteric plexus of the muscle layer.

Nitric oxide (NO), which is synthesized by the activation of NOS in the myenteric plexus, is known to be involved in vasorelaxation, neurotransmission, and inhibition of tumor cells (20). NO also regulates the accommodation reflex of the fundus and the peristaltic reflex of the intestine (21). In this study, the ingestion of *chongkukjang* mucilage facilitated an increased NOS expression in the myenteric plexus of rat GI tracts. This finding has provided the morphological evidence of NO involvement in the modulation of mobility and blood circulation in the gastrointestinal tract. This experiment also provided the first morphological evidence for the expression of uNOS- positive neurons and cells in the gastrointestinal tract after administration of *chongkukjang* mucilage.

In conclusion, the feeding of *chongkukjang* mucilage to the rats caused an increase in the immunoreactive densities of gastrin and serotonin. Increases were also observed in the immunohistochemical densities of $CD4^+$ and $CD8^+$ lymphocytes and of uNOS immunoreactive neurons and nerve fibers. This morphological evidence indicated that mucosal immune activity, gastrointestinal mobility, and blood circulation in the GI tract could be increased with the intake of *chongkukjang* mucilage.

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Table 4. Immunohistochemical density of universial nitric oxide syntase(uNOS) in gastrointestinal tract after administration of *chongkukjang* mucilage to rats for 4 weeks

		Mucosa	Submucosa	Muscularis
Stomach	Control	+	+	+
	Chongkukjang mucilage	++	++	+++
Duodenum	Control	+	+	+
	Chongkukjang mucilage	+++	++	++
Jejunum	Control	+	+	+
	Chongkukjang mucilage	+	+	+
Colon	Control	+	+	+
	Chongkukjang mucilage	++	++	+++

Immunoreactive density : + weak, ++ moderate, +++ strong

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