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Effects of *Cheonggukjang* on Immune Responses and Gastrointestinal Functions in Rats

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Abstract Effects of *cheonggukjang* on immunohistochemical reactions in gastrointestinal (GI) tract of rats were investigated. $CD4^+/CD8^+$ immunoreactive cells of *cheonggukjang*-fed diet groups were more strongly stained in lamina propria of mucosa and submucosa than those of basal diet group. Universal nitric oxide synthase immunoreactive density in colon was mildly stained in surface epithelium and mucous secretory gland, and strongly stained in submucosa and myenteric plexus in muscle layers of all *cheonggukjang*-fed diet groups. Protein kinase C-α immunoreactive cells in colons of 15 and 25% *cheonggukjang*-fed diet groups were more strongly stained in mucosa, submucosa, and muscle layers than those of basal diet group. These results indicate mucosal immune activity, gastrointestinal motility, blood circulation, and physiological activities of enteroendocrine cells in GI tract could be increased with *cheonggukjang* intake.

Keywords: *cheonggukjang*, gastrointestinal tract, immunohistochemical density, universal nitric oxide synthase, protein kinase C-α

Introduction

Propulsion of chyme or feces in the human intestine is associated with the enteric nervous system and enteroendocrine cells (1). There are several different types of enteroendocrine cells in the stomach mucosa, as well as in the epithelia of the small and large intestines. These systems produce and release polypeptides and proteins through hormonal activity. The peptides, which can be produced and released through endocrine and neural tissues, act as circulating hormones, local regulators, and neurotransmitters (2).

Lymphoid cells are associated with the gastrointestinal (GI) tract and are separated into several compartments, including lymphoid follicles, lamina propria lymphocytes, and intraepithelial lymphocytes. These cells have been found to mediate various forms of cytotoxicity (3, 4) and secrete cytokine (5) *in vitro*. They have direct contact with foreign antigens derived from gut lumen, and thus play integral roles in immune responses to these antigens and in the prevention of disease in the intestinal tract (6).

Cheonggukjang, a traditional Korean soybean-based fermented food, is produced by fermenting whole steamed soybeans with *Bacillus subtilis*, the dominant species. This product is rich in proteins and carbohydrates (7), which suggests its potential as both a nutritional and healthy food. *Cheonggukjang* is also known to have various physiological properties (8-12), such as fibrinolytic activity, anticancer activity, antihypertensive activity, hypocholesterolemic effects, hypoglycemic effects, and hypolipidemic effects.

In this study, we examined the effects of *cheonggukjang* on immunohistochemical reactions in the GI tract of rats. The immunohistochemical densities of immunocompetent cells ($CD4^+$ and $CD8^+$), universal nitric oxide synthase, and PKC-α in the GI tract were observed through immunohistochemical staining after the administration of *cheonggukjang*-supplemented diets. Effect of *cheonggukjang* intake on the modulation of the intestinal motility and mucosal immune system in the GI tract was also investigated.

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Material and Methods

Materials Traditionally manufactured *cheonggukjang* was purchased from Sunchang, Korea, freeze-dried to prepare into powder, and added to the experimental diet. The composition of *cheonggukjang* powder was 31.1% carbohydrate, 44.2% protein, 18.9% fat, and 5.8% ash.

Animals and diets Male Sprague-Dawley rats (6 weeks old, Samtako, Korea) were randomly assigned to one of four groups (n = 6/group). The experimental groups were fed one of the diets that contained 5, 15, and 25% *cheonggukjang* powder for 4 weeks. The control rats were fed a basal diet. Composition of the experimental diets of each group is shown in Table 1. The basal and experimental diets were isocaloric, and carbohydrate, protein, and fat were proportionally deducted from the experimental diet to compensate for the added *cheonggukjang* powder.

Tissue processing Six rats were sacrificed after being fed experimental diets for 4 weeks. Parts of their GI tracts were excised, fixed in 10% neutral buffered formalin, and prepared for routine paraffin embedding. Sections of tissues were cut into 7-μm slices using a microtome, mounted in slides, and stained using immunohistochemical methods.

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

Ingredients	Basal	Cheonggukjang		
		5%	15%	25%
Casein	20	18	14	10
DL-Methionine	0.3	0.3	0.3	0.3
Sucrose	55	53	49	45
Corn starch	10	10	10	10
Corn oil	5	4	2	0
AIN-76 mineral[a]	3.5	3.5	3.5	3.5
AIN-76 vitamin mix[b]	1	1	1	1
Choline bitartate	0.2	0.2	0.2	0.2
Cellulose	5	5	5	5
Cheonggukjang	0	5	15	25

[a,b]AIN-76, American Institute of Nutrition.

Immunohistochemistry Tissue sections of GI tract were deparaffined and hydrated through xylene and graded alcohol series. To quench endogenous peroxidase activity, tissue sections were incubated for 10 min in 1% H_2O_2 in phosphate buffered saline (PBS), for 1 hr in 1.5% normal goat serum in PBS, or overnight at 4°C in rat anti-CD4 (diluted 1:50, Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA), rat anti-CD8 (diluted 1:50, Santa Cruz), rabbit anti-uNOS (diluted 1:50, St. Louis, MO, USA), and rabbit anti-PKC-α (1:100, Santa Cruz) in PBS containing 0.3% Triton X-100 and 2% bovine serum albumin. After washing two times for 10 min with PBS, sections were incubated sequentially in biotinylated anti-rabbit IgG and biotinylated anti-rat IgG (Vector Lab., Burlingame, CA, USA), diluted 1:200 in the same solution as the primary antiserum for 2 hr. Subsequently, they were incubated with an avidin-biotin enzyme for 1 hr and again washed in PBS. The sections were visualized with 3,3'-diaminobenzidine (DAB) in 0.1 M Tris buffer and mounted on the gelatin-coated slides. The immunoreactions were observed under the Axioscope microscope (Carl Zeiss, Germany). Immunoreactive density was determined by comparing the stained intensity of each group after observing 10 slides per sample.

Table 2. Immunohistochemical density of $CD4^+/CD8^+$ lymphocytes in the jejunum and colon after administration of various concentrations of *cheonggukjang* in rats for 4 weeks

		Mucosa		Submucosa	
		CD4	CD8	CD4	CD8
Jejunum	Control	+	+	+	+
	5% *cheonggukjang*	+++	+++	+++	+++
	15% *cheonggukjang*	+++	++	+++	++
	25% *cheonggukjang*	++	++	++	++
Colon	Control	+	+	+	+
	5% *cheonggukjang*	+++	+++	+++	+++
	15% *cheonggukjang*	++	++	++	++
	25% *cheonggukjang*	++	+++	++	+++

Density : + weak, ++ mild, +++ strong.

Results and Discussion

$CD4^+$ and $CD8^+$ immunoreactive cells $CD4^+$ lymphocytes are located adjacent to the epithelial cells at the bottom of the crypt in the large intestine (13), while $CD8^+$ lymphocytes are preferentially located among differentiated epithelial cells. This organized cell distribution is related to the physiology of the mucosal immune system in the gut (14).

Table 2 shows the results of immunohistochemical density of $CD4^+/CD8^+$ lymphocytes in GI tracts after feeding *cheonggukjang* (5, 15, and 25%)-supplemented diets for 4 weeks. In jejunum, the basal diet group showed a few weakly stained $CD4^+/CD8^+$ immunoreactive cells in the lamina propria of mucosa and submucosa (Fig. 1a and 3a). In the case of the *cheonggukjang* diet groups, the number of $CD4^+/CD8^+$ immunoreactive cells increased considerably with the increased concentration of *cheonggukjang*, resulting in mildly stained $CD4^+/CD8^+$ cells in mucosa and submucosa for 5% *cheonggukjang*-fed diet group (Fig. 1b and 3b), strongly stained $CD4^+$ cells in lamina propria of villi (Fig. 1c), mildly stained $CD8^+$ cells in mucosal epithelium, lamina propria of villi, and submucosa for 15% *cheonggukjang*-fed diet group (Fig. 3c), and strongly and mildly stained $CD4^+/CD8^+$ cells in lamina propria of villi and mucosal epithelium for 25%

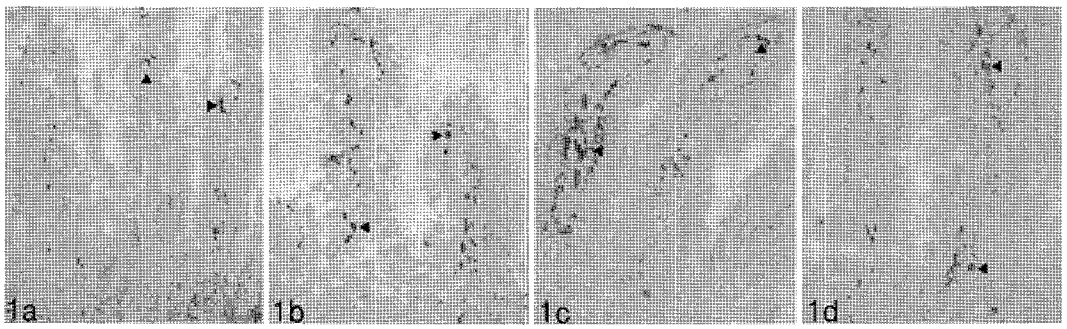

Fig. 1. Photomicrograph of $CD4^+$ immunoreactive cells in lamina propria of jejunal mucosa. 1a. Basal diet group (×100); 1b. 5% *cheonggukjang* fed diet group (×100); 1c. 15% *cheonggukjang* fed diet group (×100); 1d. 25% *cheonggukjang* fed guoup (×100).

cheonggukjang-fed diet group, respectively (Fig. 1d and 3d). In the colon, the basal diet group showed weakly stained $CD4^+/CD8^+$ immunoreactive cells in the lamina propria of mucosa and submucosa (Fig. 2a and 4a).

In the case of the *cheonggukjang* diet groups, the number of $CD4^+/CD8^+$ immunoreactive cells also increased considerably with the increased concentration of *cheonggukjang*, resulting in strongly stained $CD4^+/CD8^+$ cells in lamina propria of muosa and periphery of blood vessels in submucosa for 5% *cheonggukjang*-fed diet group (Fig. 2b and 4b), mildly stained $CD4^+/CD8^+$ cells in lamina propria of mucosa, submucosa, and periphery of blood vessels in muscle layer (Fig. 2c and 4c) for 15% *cheonggukjang*-fed diet group, and mildly stained $CD4^+$ cells in lamina propria and submucosa (Fig. 2d) and strongly stained $CD8^+$ cells in mucosal epithelium, lamina propria, submucosa, and adventitia for 25% *cheonggukjang* fed-diet group (Fig. 4d). In comparison to the basal diet group, all *cheonggukjang*-fed diet groups showed considerably increased $CD4^+/CD8^+$ immunoreactive cell numbers in mucosa and submucosa of the jejunum and colon. In the colon, protection against infection by luminal bacterial flora may represent the major function of intraepithelial cells. Analysis of Ig-secreting cells in the lamina propria of the small and large intestines revealed greater numbers of spontaneously Ig-secreting cells, particularly IgA-secreting cells, per 10^6 lamina propria lymphocytes. This may be due to the cytokine secretion profile of large intestinal IELs(intra epithelial lymphocytes), which appear to be similar to helper T cells in that they are predominantly $CD4^+$ cells (15).

According to Ishizuka and Tanaka (6), dietary fibers increase the densities of $CD8^+$ and $CD4^+$ intraepithelial lymphocytes (IELs) in the colon, and the concentrations of acetate and butyrate in cecum of rats, which result in the modulation of local proliferation in $CD8^+$ progenitor cells and promotion of homing in $CD8^+$ T cells through the large intestinal epithelium. In particular, butyrate, a major fuel for colonic epithelial cells, is known to stimulate mucosal proliferation and localize immune cells ($CD8^+$ and $CD4^+$ IELs) for the maintenance of epithelial homeostasis (16).

uNOS immunoreactive cells Nitric oxide (NO) is synthesized by nitric oxide synthase (NOS), which is involved in various physiological responses, such as vasorelaxation, neurotransmission, and inhibition of tumor cells (17). Recent pharmacological and physiological studies demonstrated that NO is a neurotransmitter in the non-adrenergic, non-cholinergic inhibitory nerves in the mammalian gastrointestinal tract and plays an important role in the neuronal regulation of the gut. On the other hand, NOS activity is present in neurons and fibers of the major enteric nerve layers in the intestine (18). NOS is a highly regulated enzyme, which catalyses oxidation of L-arginine into citrulline and nitric oxide, and is therefore used as a marker for the presence of NO.

Table 3 shows the results of immunohistochemical density of uNOS in the jejunum and colon after feeding of the *cheonggukjang* (5, 15, and 25%)-supplemented diets for 4 weeks. In jejunum, basal diet-fed group showed

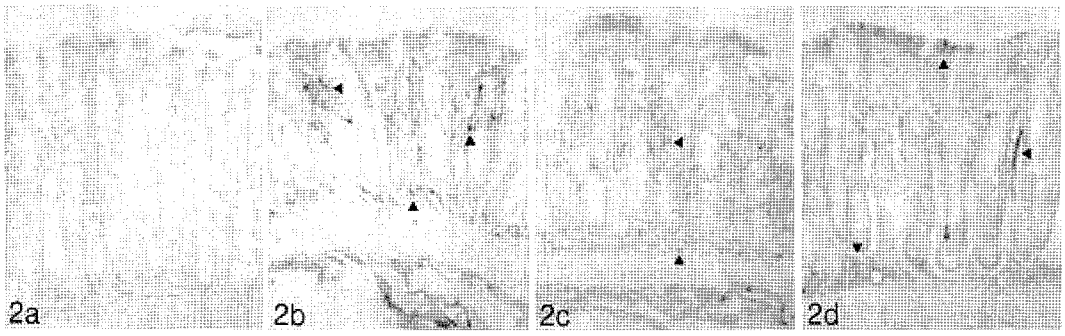

Fig. 2. Photomicrograph of $CD4^+$ immunoreactive cells in lamina propria of colonic mucosa and submucosa. 2a. Basal (×200); 2b. 5% *cheonggukjang* fed diet goup (×100); 2c. 25% *cheonggukjang* fed diet gruop; 2d. 25% *cheonggukjang* fed diet group (×100).

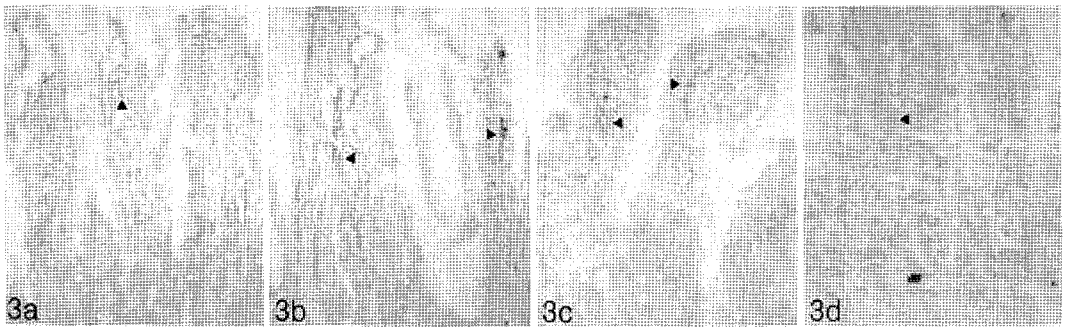

Fig. 3. Photomicrograph of $CD8^+$ immunoreactive cells in lamina propria of jejunal mucosa. 3a. Basal diet group (×100); 3b. 5% *cheonggukjang* fed diet group (×100); 3c. 15% *cheonggukjang* fed diet group (×100); 3d. 25% *cheonggukjang* fed diet group (×100).

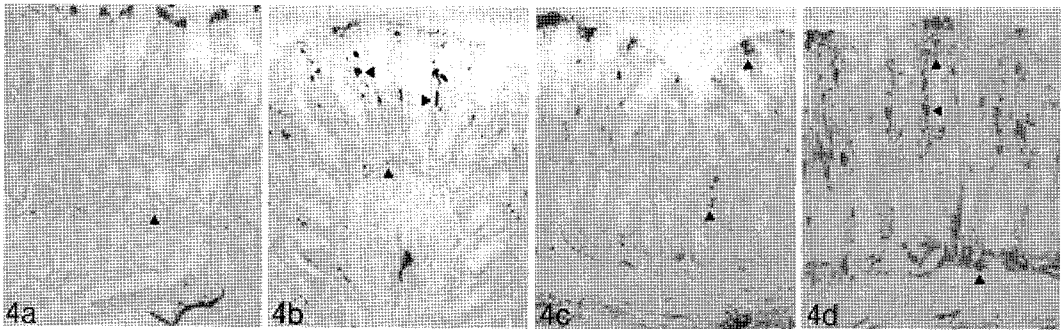

Fig. 4. Photomicrograph of $CD8^+$ immunoreactive cells in lamina propria of colonic mucosa and submucosa. 4a. Basal diet group (×100); 4b. 5% *cheonggukjang* fed diet group (×100); 4c. 15% *cheonggukjang* fed diet group; 4d. 25% *cheonggukjang* fed diet group (× 100).

weakly stained uNOS immunoreactive cells in myenteric plexus and adventitia of jejunum, whereas 5 and 25% *cheonggukjang*-fed diet groups in the surface epithelium in villi, basal portion of secretory epithelium, and lamina propria of mucosa, submucosa, myenteric plexus, and adventitia. The 15% *cheonggukjang*-fed diet group, on the other hand, had mildly stained uNOS immunoreactive cells in mucosa, submucosa, and myenteric plexus. In the colon, basal diet-fed group also showed weakly stained uNOS immuno-reactive cells in myenteric plexus and adventitia of the colon. All *cheonggukjang*-fed diet groups showed mildly stained uNOS immunoreactive cells in the surface epithelium and mucous secretory glands, and strongly stained submucosa and myenteric plexus in the muscle layers.

uNOS were widely distributed in the GI tract, with most of them located in the myenteric plexus, as well as in the submucosal plexus, mucosal epithelium, and glands (Table 3). These results indicated that the neurons may mediate majority of the inhibitory responses in the GI tract and regulate many important physiological reflexes, such as the relaxation of the lower esophageal sphincter after swallowing, receptive relaxation of the proximal stomach during eating, and descending inhibition in response to distension (19).

Protein kinase C (PKC)-α immunoreactive cells Protein kinase C (PKC) constitutes at least 11 isozymes and mediates a wide range of signals regulating cell growth and differentiation (20). Table 4 shows the results of immunohistochemical density of PKC-α in colon after feeding of the *cheonggukjang* (5, 15, and 25%)-supplemented diets for 4 weeks. The basal diet-fed group showed weakly stained PKC-α immunoreactive cells in mucosa and submucosa, and mildly stained ones in muscle layers of the colon. The 5% *cheonggukjang*-fed diet group showed strongly stained PKC-α immunoreactive cells in mucosa and mildly stained ones in submucosa and muscle layers, while 15 and 25% *cheonggukjang*-fed diet groups showed strongly stained PKC-α immunoreactive cells in mucosa, submucosa, and muscle layers. These results indicated that a significant increase in PKC-α immunoreactive cells by intake of prolonged or elevated level of *cheonggukjang* might modulate and maintain transmitter output (21).

Table 3. Immunohistochemical density of uNOS in the jejunum and colon after administration of several percentages of *cheonggukjang* in rats for 4 weeks

		Mucosa	Submucosa	Muscularis
Jejunum	Control	+	+	+
	5% *cheonggukjang*	+	+	+
	15% *cheonggukjang*	++	++	++
	25% *cheonggukjang*	+	+	+
Colon	Control	+	+	+
	5% *cheonggukjang*	++	++	+++
	15% *cheonggukjang*	++	++	+++
	25% *cheonggukjang*	++	++	+++

Density : + weak, ++ mild, +++ strong.

Table 4. Immunohistochemical density of PKC-α in the colon after administration of various concentrations of *cheonggukjang* in rats for 4 weeks

		Mucosa	Submucosa	Muscularis
PKC-α	Control	+	+	++
	5% *cheonggukjang*	+++	++	++
	15% *cheonggukjang*	+++	+++	+++
	25% *cheonggukjang*	+++	+++	+++

Density : + weak, ++ mild, +++ strong.

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