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Effect of Yam Yogurt on Colon Mucosal Tissue of Rats with Loperamide-induced Constipation

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Abstract The effects of lactic acid fermented yam yogurt (Yam/YG) on colon mucosal tissue were investigated in a loperamide-induced constipation rat models. Sprague-Dawley rats were fed for 6 weeks with 3 types of diets (normal, supplemented with lactic acid bacteria, and supplemented with Yam/YG), and were then administered loperamide intraperitoneally twice daily for 5 days. Administration of loperamide decreased fecal excretion and the moisture content of feces with increasing of numbers of pellets in the colon. On the histopathologic findings from hematoxylin and eosin (H& E) and alcian blue stainings, supplementation with Yam/YG resulted in the recovery of depleted goblet cells and mucin, and increased the numbers of Ki-67 positive cells, indicating restoration of colonic mucosa through cell proliferation and crypt regeneration against damages observed in crypt epithelial cells of loperamide-induced rats. These results indicate that Yam/YG improves evacuation and mucus production in the gastrointestinal tracts of constipated-induced rats.

Keywords: yam, lactic acid fermentation, loperamide, constipation, colonic mucus, histopathology

Introduction

In the Westernized world, lifestyle is an important determination of health later in life. A lack of physical activity, particularly if associated with over-consumption, increases the risk of development of nutrition-related diseases such as obesity, diabetes, cardiovascular disease, and constipation.

Constipation is a symptom rather than a disease, and is the most frequent digestive complaint. However, it is also one of risk factors related with colorectal cancer, and 12% of a healthy population across the world complained of constipation at some point (1). The main symptoms of constipation are straining at defecation and incomplete rectal emptying. In the first instance, treatment is usually conducted by dietary means (2). Many efforts to improve the function of the gastrointestinal tract and alleviate fecal complaints have employed live microbial supplements, based on *in vitro* studies and clinical trials (2-4).

Lactic acid bacteria as probiotic bacteria have been known to have health benefits, including enhanced intestinal microbial balance and food digestibility, control of gastrointestinal infections, and colon cancer suppression (5-8). Increasing attention has also been paid to the use of synbiotics, a combination of pre- and probiotics in a single product (9).

Yam (*Dioscorea batatas* Decne) has been used as a health food and as an ingredient of traditional Chinese herbal medicines for the treatment of anorexia, chronic diarrhea, diabetes, seminal emission, and excessive leukorrhea (10, 11). Several beneficial properties of yams have recently been reported in the literature. Yam flour has been reported to protect rats from chemical-induced toxicity (12). Dioscorin, a purified storage protein of yam, has been found to possess scavenging properties against free radicals (13). In addition, it has been found that feeding with *Dioscorea* rhizomes can improve some metabolic abnormalities, including obesity (14), gut function, and lipid metabolism (15). In recently, we found that a 40% ethanol extract of yam flour inhibited the secretion of gastric acid, and did not affect the growth of normal intestinal bacteria (16). To develop a functional food, lactic acid fermentation of yam was attempted using *Lactobacillus, Streptococcus*, and *Bifidobacterium*. The fermented yogurt was found to be acceptable to the panel through sensory evaluation (3). To evaluate the effects of yam yogurt on gastrointestinal function, we performed an animal study for 6 weeks, which resulted in improvement of gut functions as observed by the gastrointestinal transit and by lactose-producing bacteria in feces (3). The purpose of the present study was carried out to determine the gastrointestinal-promoting activity of yam yogurt through morphologic findings of colonic tissue in a loperamide-induced constipation model.

Materials and Methods

Preparation of lactic acid fermented yam yogurt Heat-dried yam powder was purchased from Bukhu Agricultural Cooperative (Andong, Korea). The composition of yam powder (g/100 g) was as follows: moisture, 3.12; protein, 4.32; crude fat, 1.31; crude ash, 2.52; crude fiber, 4.57; starch, 86.46; respectively. To prepare yam yogurt (Yam/YG), 10 g of yam powder was added at 1:10 to skim milk solution (15.4 g/100 mL) and steamed to 100°C for 50 min. After cooling to room temperature, 0.05% of the starter lactic acid bacteria (Cell Biotech Co., Gimpo, Korea) containing 10^{10} colony-forming units/mL, which was composed of *Lactobacillus acidophilus* ATCC 4356,

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Streptococcus thermophilus ATCC 19258, and *Bifidobacterium bifidus* ATCC 29521 (1:1:1) pre-cultured on *Lactobacillus* MRS broth (Difco, Detroit, MI, USA) at 37°C for 30 hr until optical density at 620 nm reached around 0.61, was inoculated and anaerobically fermented at 37°C for 20 hr.

Animal care and feeding schedule Sprague-Dawley male rats (4-5 weeks old) were purchased from Samtako, Inc. (Osan, Korea), and were subjected to a 1-week adaptation period in our lab. The rats were fed with 3 types of diets for 6 weeks; normal chow (Nestle Purina PetCare Korea, Ltd., Seoul, Korea), 10% lactic acid bacteria (yogurt)-supplemented, and 10% of Yam/YG-supplemented diets. All of the rats were fed water *ad libitum*, and were kept in a temperature-controlled environment (20 to 22°C) with an alternating photo cycle of 12 hr of light and 12 hr of darkness.

Induction of experimental constipation To verify the effect of Yam/YG on constipated colonic mucus, the animals were given 1.5 mg/kg of body weight of loperamide twice a day for 5 days. Loperamide was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in 0.9% sodium chloride. The control group was given only the vehicle in the same manner as loperamide had been administered to the experimental groups. During 5-day constipation induction, body weight and feces status were checked every day.

Histopathologic examination Specimens from each colonic segment of each rat were taken after washing and immediately immersed in 10% buffered formalin (pH 7.4) for 3 hr at room temperature, followed by a standard procedure for paraffin embedding (17). Sections were 3-5 μm thick and were stained with hematoxylin and eosin (H&E stain), and the specimens were then observed and microphotographed using a light microscope (Olympus Optical Co., Tokyo, Japan). Alcian blue staining for confirmation the amount and distribution of mucin was performed with 1% alcian blue solution (in 3% acetic acid, pH 2.5) for 2.5 hr. After rinsing with 3% acetic acid, followed by running tap water and then deionized water, the slides were oxidized in 1% periodic acid for l0 min. After rinsing in running tap water, neutral mucins were stained magenta with Schiff's reagent for 15 min. After rinsing, slides were immersed in 3 changes of 0.5% sodium metabisulphite, rinsed, and then fixed in *p*-formaldehyde vapor at 37°C for 45 min. The stained slides were mounted with gelatin. Chemicals and reagents were obtained from Sigma-Aldrich Co. (Poole, Dorset, UK) (18).

Immunohistochemistry against Ki-67 in rat colonic mucosa Immunohistochemical investigation to assess cell proliferation was performed using mouse monoclonal antibodies against Ki-67 (1:100 clone MIB-1; Dako, Carpinteria, CA, USA). Sections were deparaffinized in xylene and rehydrated in a graded series of alcohol to distilled water. Subsequently, antigen retrieval by heating in a microwave oven in 0.001 M EDTA buffer (pH 8.0) at the highest power (1,200 W) was performed for 15 min. Sections were incubated at room temperature for 1 hr with primary antibodies, and then with a peroxidase-labeled polymer (EnVision + System/HRP K4005; Dako) for 30 min. Sections were then incubated with 3, 3'-diaminobenzidine tetrachloride, counterstained with haematoxylin, and mounted using an aqueous medium. Appropriate positive and negative controls were carried out for each stain. For quantification of proliferation, 400 epithelial cells were counted for each histological section stained with anti-Ki 67 protein in 3 or 4 randomly-selected crypts. The proliferative index was defined as the ratio of Ki-67 positive nuclei to total nuclei counted and multiplied by 100. In all cases, the quantification of positively-stained cells was determined by 2 independent, experienced observers who were unaware either of the experimental conditions or the other results obtained.

Results and Discussion

Food intake and fecal moisture content The administration of loperamide significantly decreased body weight when compared with that of the control group, but a significant difference was not observed between the loperamide-administered groups (Table 1). Rats given loperamide ate significantly less food, and excreted less feces than control rats during the 5-day experiment ($p<0.05$). The fecal moisture contents in rats administered loperamide alone was approximately 13% lower than that in the control group. The mean number of fecal pellets remaining in the colons of the loperamide-administered group was approximately twice that in the control group (Table 1). These findings indicate the actual induction of constipation by loperamide, a synthetic opioid agonist drug, in the present study. Loperamide has known be

Table 1. The changes of body weight gain and stool status in loperamide-induced rats for 5 days

Group[1)]	Body weight gain (g)	Food intake (g)	Fecal excretion (g/day)	Fecal pellet number in colon	Fecal moisture content (%)
Control	11.0±2.86[a]	24.4±0.72[a]	5.7±1.38[a]	2.4±0.20[a]	55.25±7.21[a, 2)]
LPM	-2.7±1.52[b]	14.0±0.43[b]	2.9±1.12[b]	4.6±0.20[b]	46.25±9.40[b]
YG-LPM	1.2±1.93[b]	16.8±0.60[c]	4.0±1.15[a]	3.7±0.36[bc]	50.13±8.55[bc]
Yam/YG-LPM	0.8±2.51[b]	16.0±0.82[c]	4.3±1.99[a]	3.4±0.37[c]	52.87±7.45[c]

[1)]Control, Chow diet group (n=7); LPM, group administered loperamide twice daily for 5 days; YG-LPM, group fed with YG for 6 weeks followed by LPM-induced constipation; Yam/YG-LPM, group fed with Yam/YG followed by LPM-induced constipation.
[2)]Means±SE (n=7), means followed by the same letter in the column are not significantly different ($p<0.05$).

extend the evacuation time of rats (19) and inhibit colonic peristalsis (20). Accordingly, the decreased fecal excretion was not thought to be due to decreased food intake, but was more likely due to the inhibition of colonic peristalsis by loperamide (21). Supplementation with increased food intake and fecal moisture content when compared with those of rats treated with loperamide alone. Alleviation of constipation improved by may be thought to be due to a synbiotic (combined) effect of living microorganisms and yam, a soothing herb that improves the function of the gastrointestinal tract (12). Therefore, the anti-constipation effect of is ascribed to increased microbial mass (2) and stool bulk in the colon, resulting in faster transit time and, thus, easier bowel movement.

Morphologic findings of the colon Table 2 summarizes the overall data concerning histological examinations (Fig. 1) with regard to the anti-constipation-related effects of pretreatment prior to the injection of loperamide. The control group showed morphological characteristics consisting of epithelial cells with well-designated crypt and goblet cells (Fig. 1A). However, from the histopathologic findings of the loperamide alone group, crypt shortening with the depletion of goblet cells was observed to a moderate degree via H&E staining (Fig. 1B). In addition, crypt loss, inflammation, interstitial edema, and erosion were detected to a mild degree in the loperamide group. However, the extent to which the destruction of crypt and goblet cells in the yam yogurt-fed group for 6 weeks were significantly lower than that observed in the loperamide alone group (Fig. 1C). Crypt regeneration and restoration of colonic mucosa were also observed, although a limited amount of cellular inflammation of lymphocytes was seen. The Yam/YG-LPM (Group fed with for 6 weeks followed by loperamide-intraperitoneally administeration) groups maintained their morphologic integrity. Currently, little is known regarding the morphological features associated with the effect of probiotics or prebiotics on loperamide-induced colonic tissue. In general, it is known that the colon is subjected to a myriad of potentially damaging agents that may reside within the lumen for 2-3 days. Its first line of defense against these agents is the protective mucus bilayers that line the entire colonic mucosa (22). In our results, supplementation with showed a recovery of the loss and shortening of goblet cells as displayed in loperamide-induced colonic mucus, thus indicating its potential protective effect and benefits to colonic health.

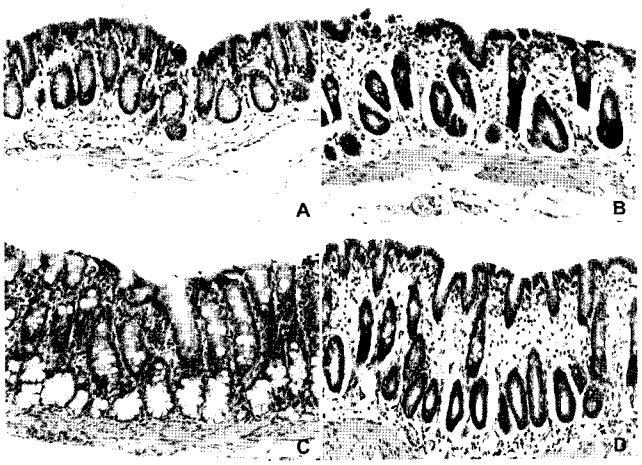

Fig. 1. Histological findings of the colon. (A) Control group. Normal colonic mucosa is present (H&E stain, 200×). (B) LPM group. The shortening and loss of crypt are present. Goblet cells are depleted (H&E stain, 200×). (C) YG-LPM group. Some crypt shortening and inflammatory lymphocytes are observed (H&E stain, 200×). (D) Yam/YG-LPM group. The shortening and loss of crypt are decreased (H&E stain, 200×).

Mucus secretion The goblet cells existed in crypt were arranged very well, and stained strongly with alcian blue at pH 2.5 in control rats (Fig. 2A), indicating that goblet cells produce normal mucin that contains sulfomucin. In contrast, the number of crypt goblet cell was significantly decreased, and these cells contained less mucin in loperamide-administered rats than in control rats (Fig. 2B). Higher numbers of goblet cells and more blue-colored goblet cells were observed in yogurt- or yam yogurt-supplemented rats when compared with the loperamide alone group, showing the increased goblet cells and intracellular mucin levels, which results in an enhancement of mucin secretion (21).

The colonic surface epithelium is a simple columnar or cuboidal epithelium that serves as a protective barrier between the host and the luminar environment and is composed of absorptive and goblet cells (23). Goblet cells synthesize, store, and secrete mucous granules (23). Mucin is a major component of luminal mucus, which protects the colorectal mucosa against mechanical and chemical damage (23). Loperamide has been shown to reduce both the synthesis and storage of mucin in crypt epithelial cells *in vivo* and *in vitro* (2, 20, 21). In our results, the increase

Table 2. Histopathologic findings of cecal tissue in loperamide-induced rats[1)]

Group[2)]	Crypt shortening	Crypt loss	IF_N[2)]	IF_L[2)]	GD[2)]	Edema	Erosion	Ulcer
Control	–	–	–	–	–	–	–	–
LPM	++	+	+	+	++	+	+	–
YG-LPM	+	–	–	+	–	–	–	–
Yam/YG-LPM	+	–	–	–	–	+	–	–

[1)] IF_N, Inflammation neutrophil; IF_L, inflammation lymphocyte; GD, goblet cell depletion. –, None; +, mild; ++, moderate.
[2)] Control, Chow diet group (n=7); LPM, group administered loperamide; YG-LPM, group fed with YG for 6 weeks followed by LPM-induced constipation for 5 days; Yam/YG-LPM, group fed with Yam/YG followed by LPM-induced constipation.

Fig. 2. Cross sections of the distal colon for mucus secretion. (A) Control group. Goblet cells in crypt are arranged very well, and are strongly stained with alcian blue at pH 2.5. Normal mucus is present (Alcian blue stain, 200×). (B) LPM group. The numbers of goblet cells are decreased (Alcian blue stain, 200×). (C) YG-LPM group. The mucus in crypt cells is mildly decreased (Alcian blue stain, 200×). (D) Yam/YG-LPM group. The mucin is filled in goblet cells (Alcian blue stain, 200×).

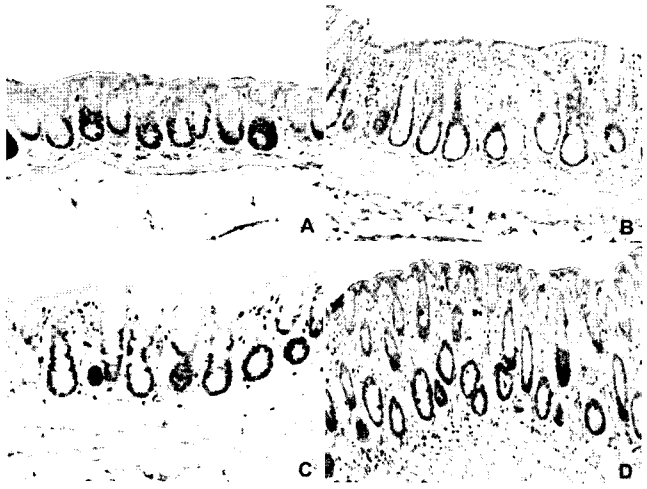

Fig. 3. Photomicrograph (200×) of colon for proliferation assessed by Ki-67. (A) the control group. Nuclei show positive staining for the Ki-67 antigen, and are located in basal zones of the crypt cells. (B) LPM group. The number of Ki-67 immunostained positive cells is decreased. (C) YG-LPM group. The number of Ki-67 positive cells is increased, and cells extend from the base to the middle zones of crypt cells. (D) Yam/YG-LPM group. The number of Ki-67 positive cells is increased.

in the number of goblet cells caused by supplementation with induced increases in luminal mucus secretion and in the thickness of the mucosa layer, leading to enhanced smooth muscle activity (24). Therefore, the improvement of mucus production by may be thought to ease the passage of feces along the colon and lessen the exposure time of the colonic mucosa to potential aggressors, thereby decreasing the risk of mucosal damage.

Cell proliferation Concerning cell proliferation, the microphotographs in Fig. 3 showed the distribution patterns of Ki-67 nuclei in the crypt cells of loperamide-induced rats with or without supplementation. The crypt cells of the control group (Fig. 3A) presented a strong and similar immunolabelling, with anti-Ki-67 antibodies showing many positive cells, whereas the number of Ki-67 antibody-positive cells in crypt cells of loperamide-administered rats was lower than those of the normal control colon group (Fig. 3B). In contrast, the number of Ki-67 immunostained positive cells was significantly increased by Yam/YG supplementation, indicating an increase of crypt cell proliferation (Fig. 3D). In the control group, proliferating cells were mainly confined to the basal zone of the crypts, whereas the proliferative cells in YG-LPM or Yam/YG-LPM groups extended from the base to the middle zones of crypts, indicating development through the continuous generation of cells in the proliferative zone (25). Proliferation and distribution of replicating cells along the length of colonic crypts are considered to be biomarkers of increased susceptibility to constipation. The result of the present study could be confirmed by the proliferative index, which showed the extent of cell proliferation based on mucosal surface and crypt localization (Fig. 4). The mean proliferative index in the LPM alone group was 35.32±1.24, and that of the Yam/YG-LPM group was 68.24±3.57, respectively. The proliferative index in the Yam/YG-LPM group dramatically increased when compared to the LPM-alone group, but without differences between the yogurt and yam yogurt-supplemented rats with loperamide-induced constipation. As suggested in the present study, increased proliferation may be a simple regenerative process, and may result in increased cell numbers with recovered morphologic features.

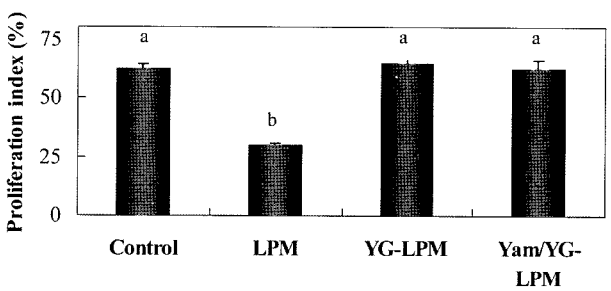

Fig. 4. Quantification of colonic epithelial cell proliferation. The values were measured by the numbers of nuclei staining positive for the Ki-67 antigen and calculated as Ki-67 positive nuclei/total nuclei × 100. Means±SE (n=7), Means followed by the same letter in the column are not significantly different ($p< 0.05$).

In conclusion, Yam/YG serves as a lubricant as confirmed by a higher level of daily fecal excretion, higher water content of fecal pellets, and fewer fecal pellets remaining in the colon when compared with those of the loperamide-administered group. Histopathologic findings indicate that supplementation with prevents the depletion of goblet cells and the reduction of mucin synthesis, and increased the numbers of Ki-67 positive cells in crypt

goblet cells, indicating restoration of colonic mucosa through cell proliferation and crypt regeneration against damages observed in crypt epithelial cells of loperamide-induced rats. The above results indicated the possibility that supplementation with can prevent the reduction in mucus secretion from the colon that accompanies constipation, and can prevent the loss of the ability to protect against mechanical or chemical injury.

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