Histological alterations of intestinal villi and epithelial cells after feeding dietary sugar cane extract in piglets

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Introduction

The weaning of piglets at three to four weeks of age is thought to be the most critical period in the life of pigs, because the piglets are subjected to stressors such as environmental, nutritional and microbial unbalances (Nabuurs, 1998). Weaning induces decreased feed intake, impaired intestinal morphology and function, diarrhea, and decreased growth immediately after weaning (Pluske et al., 1995, 1997). To solve these problems, antibiotics (Kyrilakis et al., 1997) and probiotics (Tortuero, 1973; Han et al., 1984; Kalbende et al., 1992) have been used as feed supplements to promote growth performance and to control disease. The facts that using antibiotics produces resistant microorganisms to antibiotics (Newman et al., 1990) and that some probiotics have no effect on improving body weight gain (Watkins and Kratzer, 1983; Watkins and Kratzer, 1984; Maiolino et al., 1992) suggest that an alternative to using antibiotic and probiotic growth promotants is needed to obtain consistent results. Some dietary ingredients such as organic acid, zinc and copper have also tried to counteract postweaning problems. Dietary organic acids improved the growth performance of weaned piglets (Partanen and Mroz, 1999). Feeding high dietary concentrations of zinc and copper stimulated growth of weanling pigs (Hill et al., 2000). As another growth promotant, sugar cane extract (SCE) might have a possibility to solve postweaning problems, because the SCE has growth-promotion effect (El-Abasy et al., 2002; El-Abasy et al., 2004).

SCE is a byproduct of removing glucose, fructose and sucrose from sugar cane juice produced from sugar cane in the raw sugar manufacturing process. SCE has functions such as immuno-stimulation (El-Abasy et al., 2003) and growth-promotion (El-Abasy et al., 2002; El-Abasy et al., 2004). These functions seem to be induced by increased function of intestine, because the dietary SCE caused the hypertrophied intestinal villi and epithelial cells on the villus apical surface in chickens (Yamauchi et al., 2006a,b). The intestine is well known to be direct organ for digestion, absorption and immunity of ingested feed, and intestinal histological alterations were induced by the diets fed to the animals (Langhout et al., 1999; Yasar and Forbes, 1999). Increased values of villus height and cell mitosis numbers as well as protuberated cells were found in activated function of intestine (Yamauchi et al., 2010). Therefore, intestinal histology is thought to be able to assess the supplemented feed ingredients.

Chickens and pigs are monogastric animals, and have similar intestinal histology. After feeding charcoal powder (including wood vinegar compound liquid), increased intestinal villus height and protuberated cells on the villus apical surface were found both in chickens (Samanya and Yamauchi, 2001) and pigs (Mekbungwan et al., 2004). As the hypertrophied intestinal histologies were observed in chickens after feeding dietary SCE (Yamauchi et al., 2006a), the present feeding SCE on pigs would also induce almost similar intestinal histological alterations.

In this study, the effects of 0.05 or 0.1% dietary SCE on intestinal histology in piglets were observed using light and scanning electron microscopy to obtain a basal data. In addition, growth performance was compared among groups.

Materials and methods

Sugar cane extract preparation
SCE (169 g/kg CP, 5 g/kg fat, 361 g/kg ash) was produced from sugar cane (Saccharum officinarum L.) in the raw sugar manufacturing process by Shin Mitsui Sugar Co., Ltd. (Tokyo, Japan) as follows: most of sugar com-
components such as glucose, fructose and sucrose from sugar cane juice were separated by an ion exchange column chromatography using synthetic adsorbent to produce SCE. Then this SCE was adsorbed to oilcake of rice bran (on dry matter, 1:4) and dried for dietary supplement (Table 1).

Animals and housing

Feeding experiments were carried out two times, in spring (6 piglets) and summer (6 piglets) (total 12 piglets). For each season, 6 commercial crossing castrated male piglets [(Large white × Landrace) × Duroc], were weaned at the age of 26 days with an average body weight of 8 kg. Piglets were allotted to three groups of two animals; a control group was fed the basal diet and the other groups were fed the basal diet supplemented with 0.05 or 0.1% SCE. The two pigs of each treatment were housed in wire pens (1.5 m × 1.0 m) under daily lighting regimen of 24 h of light and environmental room temperature. Each pen had a feeder and a water nipple to ensure ad libitum feeding and free water access. A commercial basal diet was pre-starter diet (Just one starter®) for first two weeks, and starter diet (We milk finisher®) for the final colonic junction. The middle part of the approximately 20 cm proximal to the ileo-caecal junction was taken about 30 min after the piglets were euthanized. Five commercial crossing castrated male piglets (total 12 piglets). For each season, 6 piglets (final body weight average was 22.10, 22.65, 23.00 kg for each feeding experiment, piglets (final body weight were measured weekly. At the end of ing experimental period, feed intake and body weight were measured weekly. At the end of each feeding experiment, piglets (final body weight average was 22.10, 22.65, 23.00 kg for control, 0.05% SCE and 0.1% SCE groups, respectively) were taken to a slaughterhouse; each piglet was anaesthetised with a 15 mL somunopenchi (64.8 mg/ml pentobarbital sodium) (Kyoritsuseiyaku Co. Ltd., Tokyo Japan) by intravenous injection and exsanguinated. All experimental procedure was carried out according to the humane care guidelines for the care and use of laboratory animals established by the Mitsui Sugar Co. Ltd.

Tissue sampling

Four pigs from each of the three groups were used for tissue sampling. The abdominal cavity was opened along the midline immediately after the piglets were euthanized. Five cm sample of the duodenum was taken about 20 cm caudal to the stomach and of the ileum approximately 20 cm proximal to the ileocolonic junction. The middle part of the remaining small intestine was regarded as the jejunum. Each sample was ligated with a thread at both ends and a mixture of 30 mL glutaraldehyde and 40 mL paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) was injected. Then, each sample was removed from the abdominal cavity, kept in a bottle with the same fixative, and prepared for light and scanning electron microscopy.

Light microscopy

A 2 × 3 cm segment from each 5-cm intestinal segment was fixed with Bouin’s fixative solution for one week at room temperature, embedded in paraplast and cut into 5 µm cross sections. Every 10th section was collected and stained with hematoxylin-eosin. For villus height measurement, the villi including the lamina propria were chosen and the length from the villus tip to the bottom excluding the intestinal crypt was measured. Two villi were selected under 10×4 magnification for each section. Sixteen values of villus height were counted from 8 sections per pig, and the average of these values was expressed as the mean villus height for each pig. To measure villus area, the width of villus was measured at the basal and apical parts. Two villi were selected under 10×4 magnification for each section. Sixteen samples were counted from 8 sections per pig. The apparent villus area was calculated from the villus height, basal width and apical width. The average of these values was expressed as the mean villus height for each pig.

Table 1. Feed ingredients and chemical composition of basal commercial pre-starter and starter mush diets of piglets.

| Ingredients, % | Pre-starter* | Starter# |
|---------------|-------------|----------|
| Animal feed | 40.0 | 5.0 |
| (dried skim milk and whey, fish meal, blood plasma) | | (concentration whey protein) |
| Cereal grains | 34.0 | 60.0 |
| (wheat flour, cooked corn, bread crumbs, soybean flour) | | cooked corn, corn, wheat flour |
| Plant oil cake | 3.0 | 19.0 |
| (dehulled soybean oil cake) | | soy bean oil cake |
| (soy bean oil cake) | | (corn germ meal) |
| Others | 23.0 | 16.0 |
| tallow, cake waste, lactose, glucose, sugar, calcium carbonate, calcium phosphate, lactic acid fermentation culture medium, bread yeast fermentation culture medium, asparagus fermentation culture medium, diatomite, thiamin, loricirice extraction by-product, fructo-oligosaccharide syrup, egg white powder, enzymatic treatment palm seedcake, citric acid, lactic acid, light liquid paraffin, yeast for feed, loricirice extraction, stevia, silicic anhydride, bentonite |
| Chemical components | | |
| Crude protein, % | 22.0 | 17.0 |
| Crude fibre, % | 7.0 | 4.0 |
| Crude fat, % | 3.0 | 3.5 |
| Crude ash, % | 9.0 | 7.0 |
| Calcium, % | 0.8 | 0.55 |
| Phosphorus, % | 0.5 | 0.35 |
| Total digestible nutrients, % | 80.0 | 80.0 |
| Digestible energy, Mcal/kg | 3.88 | 3.53 |

*Concentrate mixture including: asialamycin, 40 g titrate/ton; citric acid morand teru, 30 g/ton; fumaric acid (as fumaric acid 0.09%); propionic acid (as propionic acid 0.002%); formic acid (as formic acid 0.0014%; vitamin A; vitamin D3; vitamin E; vitamin K; vitamin C; vitamin B1; vitamin B6; vitamin B12; vitamin B5; vitamin B2; vitamin B9; vitamin B3; pantothenic acid; niacin; colin; folc acid; biotin; manganese sulfate; zinc sulfate; ferrous sulfate; cobalt sulfate; sulfite; iodine acid calcium; peptide manganese; peptide zinc; peptide copper; peptide iron; lysin; threonine; methionine; tryptophan; L-valine; L-arginine; ethionin; Bacillus subtilis; sodium glutamate; saccharin sodium; protein splitting enzyme; amyloptic enzyme; fibre splitting enzyme; β-gluconase; flavoring agent; gluconic acid sodium. #Concentrate mixture including: nosiheptide, 8 g titre/ton; citric acid morand teru, 30 g/ton; fumaric acid (as fumaric acid 0.09%); propionic acid (as propionic acid 0.002%); formic acid (as formic acid 0.0014%); vitamin A; vitamin D3; vitamin E; vitamin K; vitamin C; vitamin B1; vitamin B6; vitamin B12; vitamin B5; vitamin B2; vitamin B9; vitamin B3; pantothenic acid; niacin; colin; folc acid; biotin; manganese sulfate; zinc sulfate; ferrous sulfate; cobalt sulfate; sulfite; iodine acid calcium; peptide manganese; peptide zinc; peptide copper; peptide iron; lysin; threonine; methionine; tryptophan; L-valine; L-arginine; ethionin; Bacillus subtilis; sodium glutamate; saccharin sodium; protein splitting enzyme; amyloptic enzyme; fibre splitting enzyme; β-gluconase; flavoring agent; fibre splitting enzyme; amyloptic enzyme; protein splitting enzyme; β-gluconase, ethionin.
values was expressed as the mean villus area for each piglet.

To measure one cell area on the 5-µm cross section, the area of the epithelial cell layer was randomly measured in the middle of the villi and the number of cell nuclei within this layer was counted. The area of the epithelial cell layer was then divided by this number. This measurement was employed in one to two fields per section. Sixteen samples were counted from 8 sections per piglet, and the average of these values was expressed as the mean cell area for each piglet.

To measure the cell mitosis number per crypt, five crypts with almost the same size as one microscopic field (10×40 magnification) were randomly selected, the mitosis numbers were counted and then expressed as cell mitosis per one crypt. One to two fields per section were measured and 16 cell mitosis numbers were counted from 8 sections per piglet, and an average of these values was expressed as a mean cell mitosis number for each piglet. These measurements were recorded using an image analyser (Nikon Labophot-2, Nikon Co., Ltd., Tokyo, Japan). Finally, the mean of each intestinal parameter from the respective four piglets was expressed as the mean villus height, villus area, cell area and cell mitosis for one group.

**Scanning electron microscopy**

A 4×5 mm segment from the 5-cm duodenal segment close to the light microscopic sample was cut, and slit longitudinally along the non-mesenteric side for its entire length. The intestinal contents were washed with 0.01 M phosphate-buffered saline (pH 7.4). The tissue samples were pinned flat to prevent curling and fixed vertically with the mucosal surface facing downwards in a fixative mixture of 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 1 h. The tissue block was further cut into a 4×7 mm rectangle and fixed for an additional 1 h. The pieces were rinsed with 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed with 10 g/L osmium tetroxide in ice-cold buffer for 2 h. The specimens were dried in a critical-point drying apparatus (Hitachi Freeze Dryer, Hitachi Ltd., Tokyo, Japan). The dried specimens were coated with platinum (Hitachi E-1030 Ion Sputter, Hitachi Ltd.) and observed with a scanning electron microscope (Hitachi S-4300SE/N, Hitachi Ltd.).

**Statistical analysis**

From the viewpoint of animal welfare, three animals are known to be enough for histological observations. In this study, we used four
piglets. All data collected for growth performance and light microscopic examination were statistically analyzed by using the one-way analysis of variance (ANOVA), and significant differences between the treatments were determined with Duncan’s multiple range test using the SAS® program (SAS Institute, Inc., Cary, NC, USA). Differences at P<0.05 were considered as significant.

Results

Feed intake (43.15, 44.57, 44.85 kg for control, 0.05% SCE and 0.10% SCE groups, respectively), body weight gain (13.99, 14.42, 14.83 kg for control, 0.05% SCE and 0.10% SCE groups), and feed efficiency (0.324, 0.323, 0.330 for control, 0.05% SCE and 0.10% SCE groups) did not show a difference among groups.

Most of the values for villus height, villus area, cell area and cell mitosis numbers were not different among groups, except for that the villus area of the 0.10% SCE group and the cell area of both SCE groups increased significantly compared to the control (P<0.05) (Figure 1). In regard to cell mitosis, the 0.10% SCE group was higher numbers than the 0.05% SCE group.

The duodenal apical surface of the control had flat cells (Figure 2A, long arrows). In the 0.05% (Figure 2B) and the 0.10% (Figure 2C) SCE groups, many clearly protuberated cells were found (short arrows). In addition, in the 0.05% SCE group, deeper cells at the sites of recently exfoliated cells (stars) frequently appeared. The jejunal apical surface of the control had flat cells, showing a smooth surface (Figure 3A). In the 0.05% (Figure 3B) and 0.10% (Figure 3C) SCE groups, cell clusters (arrow heads) aggregated by many protuberated cells were shown to have developed in addition to individual protuberated cells. These cell clusters were much more developed in the 0.05% SCE group than in the 0.10% SCE group. The ileal apical surface of the control (Figure 4A) had flat cells, but the 0.05% (Figure 4B) and 0.10% SCE (Figure 4C) groups had many protuberated cells.

Discussion

The main aim of this study was to demonstrate that dietary SCE can induce hypertrophied intestinal villi and epithelial cells. Long intestinal villi mean a greater surface area for nutrient absorption (Onderci et al., 2006). Greater intestinal villus height and increased cell mitosis numbers are indicators that the function of the intestinal villi was hypertrophied (Langhout et al., 1999; Yasar and Forbes, 1999). Furthermore, increased villus size was also related to raised cell proliferation in the crypt (Lauronen et al., 2000) and provided more surface area for nutrient absorption and thus improved nutrient digestibility (Onderci et al., 2006). In the present study, the villus height, villus area, and cell area of the duode-
num and jejunum were slightly improved with increased SCE levels, although no significantly increased. The villus area and cell area of the 0.10% SCE group were significantly higher than those of the control. The cell mitosis numbers for the 0.10% SCE group were much greater than those of the 0.05% SCE group. These results suggest that the intestinal villi might be slightly hypertrophied by dietary SCE, especially in the 0.10% SCE group. On the other hand, epithelial cells on the villus apical surface of the SCE groups developed into protuberated cells, and many more developed into cell clusters in the 0.05% SCE group. Cellular protrusion features demonstrated that cellular functions were activated (Yamauchi et al., 2006b). Such cell protrusion features were also seen in chickens (Kambualai et al., 2010) and in pigs (Mekbungwan et al., 2008). In addition, cell clusters were observed in chickens fed SCE (Kambualai et al., 2010). These data indicate that epithelial cells on the villus apical surface would be hypertrophied by the dietary SCE, especially in the 0.05% SCE group. It is not clear at present why hypertrophied epithelial cells developed in the SCE group, but it is possibly related to a certain ingredient of SCE. Further study is being carried out to find such a component from SCE. Besides, small particle feed ingredient such as semi-purified pellet diet can easily induce hypertrophic intestinal alteration at epithelial level than villus level (Maneewan and Yamauchi, 2003).

Conclusions

The 0.10% SCE, having higher values of light microscopic parameters, may effectively raise the villus function. The 0.05% SCE group, having much more protuberated cells and cell clusters, may effectively activate the cellular function. Using SCE in weaning piglets appears to have an effect on the maintenance of intestinal health.

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