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Morphological Demonstration of the Stimulative Effects of Charcoal Powder Including Wood Vinegar Compound Solution on Growth Performance and Intestinal Villus Histology in Chickens

Mongkol Samanya and Koh-en Yamauchi*

Laboratory of Animal Science, Faculty of Agriculture, Kagawa University, Miki-Cho, Kagawa-ken, 761-0795, Japan

We investigated which of the following substances is most effective for inducing improved growth performance and activated intestinal villus function : a wood vinegar compound solution (WVC), a charcoal powder, or a mixed powder of amorphous charcoal powder and WVC (CWVC). Cockerels (Julia strain) were fed ad libitum the following treatment diets for 28 d : 1) a commercial basal diet (CP, 16% ; ME, 2,800 kcal/kg), 2) 0.1% WVC in water, 3) 1% dietary charcoal powder diet, and 4) 1% dietary CWVC (CP, 2.5%) diet. After the end of feeding experiment, each intestinal segment was examined by light and scanning electron microscopy for morphological changes in the villi.

Although feed intake and body weight gain were not significantly different among feeding experimental groups, they tended to be increased in all experimental groups than those of control after feeding each experimental substance. In the feed conversion ratio, CWVC group showed the lowest value.

Although intestinal villus height, epithelial cell area and cell mitosis number did not show a significant difference among feeding experimental groups, these parameters tended to be more increased in all experimental groups than those of control. In CWVC group, cell area of the jejunum and ileum were significantly elevated ($P<0.05$).

On the duodenal villus surface of WVC and charcoal groups, some cells devoid of microvilli were observed. In the CWVC group, such damaged cells were not found, and more remarkable cell protuberances than those of the control group appeared, suggesting that duodenal villus function might be activated. Such an activated morphology on the villus tip surface was found in all experimental groups in the jejunum, but in the charcoal and CWVC groups in the ileum.

The present villus morphological findings demonstrate that among WVC, charcoal and CWVC, the dietary supplement of CWVC might be most effective substance for activating the intestinal absorptive function, and that the functional activation of whole intestine including the ileum may induce a slight elevation of chicken growth performance.

Key words : charcoal including wood vinegar compounds, villi, morphological demonstration, electron microscope

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* Corresponding author : Koh-en Yamauchi. TEL/FAX : 087-891-3053, E-mail : yamauchi@ag.kagawa-u.ac.jp

Introduction

As some kinds of toxins are not heat-labile, they can survive the heating process during manufacture of chicken feeds. In addition, vaccination against these toxins is impossible due to the nonantigenic nature of these compounds. Consequently, in spite of the extensive use of disinfectants and insecticides, it may not be possible to completely prevent toxic and fungal contamination of chicken diets. At present, non-nutritive sorptive substances are known to elevate the plant production and have shown some promise in reducing the effects of toxins on chicken growth performance. The promoting effects of wood vinegar compound solution (WVC), a by-product solution from charcoal production, have been reported in mycelial growth of *Pleurotus ostreatus* and *Rhizopogon rubescens* (Yoshimura and Hayakawa, 1993) and in fruiting of *Pleurotus ostreatus* (Yoshimura *et al.*, 1995). Dietary WVC enhanced intestinal calcium absorption in rats (Kishi *et al.*, 1999). On the other hand, charcoal is a solid fuel made by dry distillation of wood, the powder of which is traditionally scattered on the floor in animal housing to reduce the smell of feces by adsorbing ammonia. Recently, charcoal has been used as an oral antidote to reduce the absorption of poison from the gastrointestinal tract, because charcoal acts as an insoluble carrier that nonspecifically adsorbs molecules, thereby preventing their absorption. When activated charcoal was added into diets containing aflatoxins or T-2 toxin, the reductions of feed intake and body weight gain of chickens tended to be ameliorated (Anjaneyulu *et al.*, 1993 ; Dalvi and Ademoyero, 1984 ; Dalvi and McGowan, 1984 ; Edrington *et al.*, 1997).

In addition to these effects of WVC and charcoal, a mixed powder of amorphous charcoal carbon powder and WVC (CWVC) has also been shown to induce a significant increase in hen-day egg production and feed conversion ratio (Sakaida *et al.*, 1987a) and in broiler hatchability (Sakaida *et al.*, 1987b). Also in a previous study, improved feed conversion ratio and activated morphological changes of intestinal villi were observed in chickens fed a 1% CWVC diet (Samanya and Yamauchi, 2001). As CWVC is a mixture of charcoal and WVC, it is not yet clear whether these improved results were due directly to an effect of one of them or to a combined effect of both. The purpose of this experiment was, therefore, to try and resolve this question by comparing birds fed WVC or charcoal with birds fed CWVC, and then to investigate which substances of them induce such growth performance and morphological changes of intestinal villi.

In this study, we examined effects of WVC, charcoal and CWVC on feed intake, body weight gain, and feed conversion ratio in chickens. Then, light microscopic observations of villus height, cell area, and cell mitosis number and scanning electron microscopic alterations of villus tip surface were compared in each intestinal segment of each group.

Materials and Methods

Birds and experimental design

Male Single Comb White Leghorn chickens (*Gallus gallus domesticus*) (Julia

Table 1. Composition of basal diet (air dry basis)

Ingredients and nutrients	%
Ground corn+milo	66
Soybean meal	17
Rice bran	8
Fish meal	6
Concentrate mixture[1]	3
Crude protein	16
Crude fat	2
Crude fiber	8
Ash	9
Calcium	0.55
Phosphorus	0.45
Metabolizable energy (kcal/kg)	2,800
Lysine	0.56
Methionine	0.25
Methionine+cystine	0.53
Tryptophan	0.14
Threonine	0.55

[1] Including 3% premix.
Premix provided the following per kg of diet : vitamin A, 2.6 IU ; vitamin D_3, 7 IU ; vitamin E, 2.5 mg ; vitamin K_3, 3 mg ; vitamin B_1, 2 mg ; vitamin B_2, 1.5 mg ; vitamin B_6, 2 mg ; vitamin B_{12}, 0.003 mg ; biotin, 2 mg ; folic acid, 2 mg ; pantothenate, 1.5 mg ; niacin, 2 mg ; choline, 2 mg ; iodine, 3 mg ; manganese, 2 mg ; ferrous, 4 mg ; zinc, 2 mg ; copper, 2 mg.

strain) were allotted to individual cages in an environmentally controlled room with a 13-h photoperiod (06 : 00 to 19 : 00 h) and a mean temperature of 25℃. At 19 w of age, 28 birds were randomly divided into 4 groups of 7 birds each, as follows : 1) intact control chickens fed on a conventional finisher mash diet (pH, 6.07 ; Table 1 ; Nippon Formula Feed Manufacturing Company, Ltd., Kanagawa, Japan), 2) 0.1% WVC in water (pH, 6.06), 3) 1% charcoal powder in the basal diet (pH, 6.03), and 4) 1% CWVC (pH, 6.07 ; Nekkarich®, Miyazaki Midori Seiyaku Co., Ltd, Miyazaki, Japan ; CP, 2.5% ; crude fiber, 0.3% ; ash, 14.4% ; Ca, 3.9% ; P, 0.2% ; water, 28.6%) in the basal diet. These non-nutritive sorptive materials were prepared by a commercial company. Birds were given access ad libitum to water and each experimental diet for 28 d. Feed intake and body weight gain were measured weekly.

Tissue sampling

At the end of the experiment 4 birds randomly selected from each group were decapitated under light anesthesia with diethyl ether. All experimental treatments were performed according to the humane care guidelines provided by the Faculty of

Agriculture of Kagawa University. Immediately after killing the birds, the whole small intestine was removed and put into a mixture of 3% glutaraldehyde and 4% paraformaldehyde fixative solution in 0.1 M cacodylate buffer (pH 7.4). The same fixative was also injected into the intestinal lumen. The duodenum (the ventriculus to pancreatic and bile ducts), jejunum (bile ducts to Meckel's diverticulum) and ileum (diverticulum to ileo-cecal-colonic junction) were separated. The middle part of each intestinal segment was taken for tissue sample.

Light microscopic examination

In the fixative solution described above, each intestinal segment was cut at 1-cm length, fixed with Bouin's solution and embedded in paraplast. Cross-sections were made at 5-μm thickness and stained with hematoxylin-eosin for light microscopy.

For the villus height measurement, all villi having the lamina propria were measured from villus tip to the base, excluding the crypt in one transverse section. An average of these values was expressed as a mean villus height per section. A total of 8 sections were counted from one bird, and an average of 8 villi heights per each section expressed as the mean villus height for each bird. Finally, these 4 mean villus heights from 4 birds were expressed as the mean villus height per group.

For the cell area, the epithelial cell layer was randomly measured at the middle part of the villi on a 5-μm transverse section. Next, the number of cell nuclei within this measured cell layer was counted. Finally, the area of epithelial cell layer was divided by the number of cell nuclei to obtain an epithelial cell area. Two cell areas were measured from one transverse section, and an average of these two values, expressed as the mean cell area per one section. A total of 8 sections were counted from one bird, and an average of 8 cell areas per each section was also expressed as the mean cell area for each bird. Finally, these 4 mean cell areas from 4 birds were expressed as the mean cell area for one group.

For cell mitosis number, mitotic cells having a homogenous, intensely hematoxylin stained basophilic nuclei were counted as cell mitosis numbers as previously described (Tarachai and Yamauchi, 2000). All cell mitoses numbers of the crypt observed in one transverse section were counted. Total number of cell mitoses was calculated from 5 different sections for each bird, and these 5 values were used to calculate a mean cell mitosis for one bird. Finally, these 4 mean cell mitoses from 4 birds were expressed as the mean cell mitosis in one group.

Statistical analysis

The values of villus height, cell area and cell mitosis number were measured using an image analyzer (Nikon Cosmozone 1S, Nikon Co., Tokyo, Japan). The average values of each parameter of each bird from each group were analyzed across all treatment groups by one-way analysis with a Duncan's multiple range test using Stat View program (Abacus Concepts, Inc., HULINKS, Inc., Tokyo, Japan). Differences at $P<0.05$ were considered significant.

Scanning electron microscopic examination

For scanning electron microscopic observations, a 2-cm tissue sample of each intestinal segment lying next to the sample for the light microscopic examination was

transversely cut, slit longitudinally, and intestinal contents was removed by washing with 0.01 M phosphate-buffered saline (pH 7.4). To prevent the outward curling of the slit intestine, tissue samples were pinned flat, serosa side up, to the paraffin-covered bottom of a Petri dish containing a mixture of 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 1 h. Then, samples were cut into 4 mm × 7 mm pieces and fixed for 1 h more. These pieces were postfixed with 1% osmium tetroxide in ice-cold buffer for 2 h and dried in a critical point drying apparatus (Hitachi HCP-1, Hitachi Ltd., Tokyo, 100-8220 Japan) using liquid carbon dioxide as the medium. After coating with platinum (RMC-Eiko RE vacuum coater, Eiko Engineering Co., Ltd., Tokyo, Japan) at 100 millitorr under 7 milliamperes for 15 min, all villi in the pieces were observed using a scanning electron microscope (Hitachi S-800, Hitachi Ltd., Tokyo, Japan) at 8 kV.

Results

Feed intake, body weight gain, and feed conversion ratio

Mean water intake (bird/week) of control, WVC, charcoal and CWVC groups was 121, 141, 155 and 161 m*l*, respectively. Table 2 shows growth performance of control and chickens fed these dietary non-nutritive sorptive material diets. Although feed intake and body weight gain were not significantly different among feeding experimental groups, they tended to be increased in all experimental groups than those of control after feeding each experimental substance. In the feed conversion ratio, CWVC group showed the lowest value.

Villus height, cell area, and cell mitosis

Table 2. Effects of conventional, dietary wood vinegar compound water (WVC), charcoal powder and charcoal powder with wood vinegar compound diets (CWVC) on feed intake, body weight gain and feed conversion ratio in chickens (means±SE, n=7)

Items	Conventional	WVC	Charcoal	CWVC
Feed intake (g/bird/day)				
Week 1	65.21± 2.02	69.4 ± 5.72	58.65± 4.47	66.28± 5.22
Week 2	53.22± 4.05	65.35± 4.12	61.41± 3.09	62.95± 1.85
Week 3	60.81± 2.2	65.38± 3.27	63.56± 1.9	63.8 ± 1.91
Week 4	67.57± 2.54	69.82± 2.71	69.51± 3.32	71.71± 5.39
Total feed intake (g/bird)	1,727.8 ±55.5	1,889.8 ±51.1	1,771.6 ±51.1	1,853.1 ±91.8
Body weight gain (g/bird/day)				
Week 1	7.96± 1.28	8.97± 1.15	5.46± 2.24	9.76± 1.86
Week 2	4.49± 1.4	7.14± 1.32	9.18± 1.31	7.14± 0.97
Week 3	9.18± 0.93	8.98± 0.81	9.18± 1.12	9.04± 1.5
Week 4	8.36± 1.05	5.1 ± 0.76	8.57± 1.6	8.09± 2.38
Total body weight gain (g/bird)	210±17.45	211.42±17.2	227.1 ±19.43	238.33±32.7
Feed conversion ratio (bird)	8.44± 0.47	9.12± 0.42	8.07± 0.56	8.01± 0.67

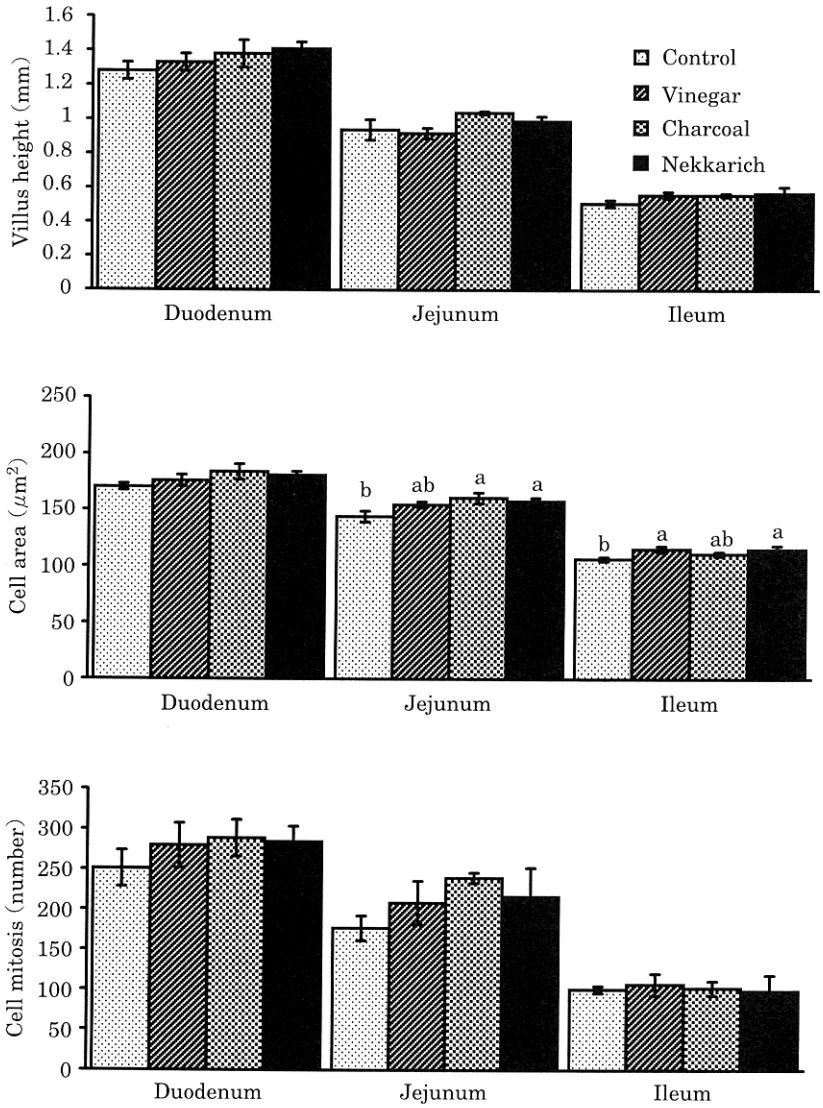

Fig. 1. Villus height, cell area, and cell mitosis number in the crypt of the duodenum, jejunum, and ileum in conventional layer finisher diet (conventional), 0.1% wood vinegar compound solution (WVC), 1% charcoal powder diet (charcoal), and 1% charcoal powder with wood vinegar compound diet (CWVC). Note that all parameters in all experimental substance groups tend to show an increased value than those of conventional group.
[a,b] Means with different superscripts are significantly different from each other ($P<0.05$) (means±SE ; n=7).

Fig. 1 shows villus height, cell area and cell mitosis number in each intestinal segment of control, WVC, charcoal and CWVC groups. Although these histological parameters did not show a significant difference among feeding experimental groups, they tended to be more increased in experimental groups than those of control group,

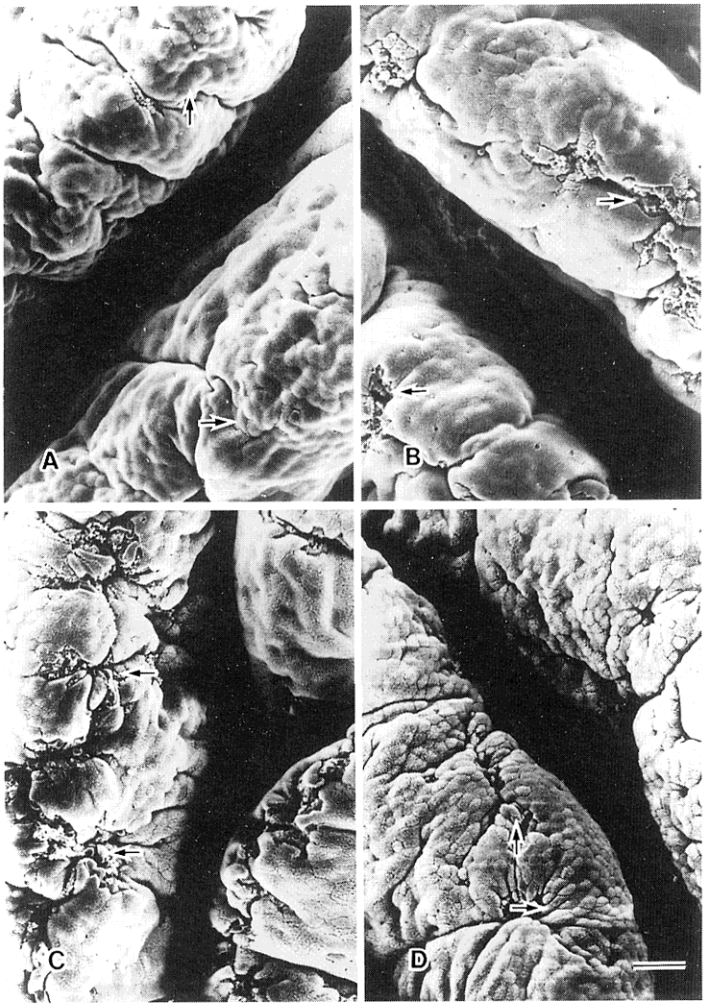

Fig. 2. Duodenal villus surface in chickens given conventional diet (A ; arrows, slight cell protuberance), 0.1% wood vinegar compound solution (B ; arrows, cells devoid of microvilli), 1% charcoal powder diet (C ; arrows, cells devoid of microvilli), and 1% charcoal powder with wood vinegar compound diet (D ; arrows, clear cell protuberance). Note the activated morphological changes after feeding the charcoal powder with wood vinegar compound diet. Scale bar=23μm, (x 290).

and cell area was significantly increased in the jejunum of charcoal and CWVC groups and in the ileum of WVC and CWVC groups ($P<0.05$).

Alterations of the villus surface

In the duodenum, the villus apex of the control (Fig. 2A) revealed a faint cell outline between each epithelial cell due to its protuberance into the lumen (arrows). At the exfoliative zone on the villus tip of WVC (Fig. 2B) and charcoal (Fig. 2C) groups, some cells devoid of any microvilli (arrows) were appeared among the intact

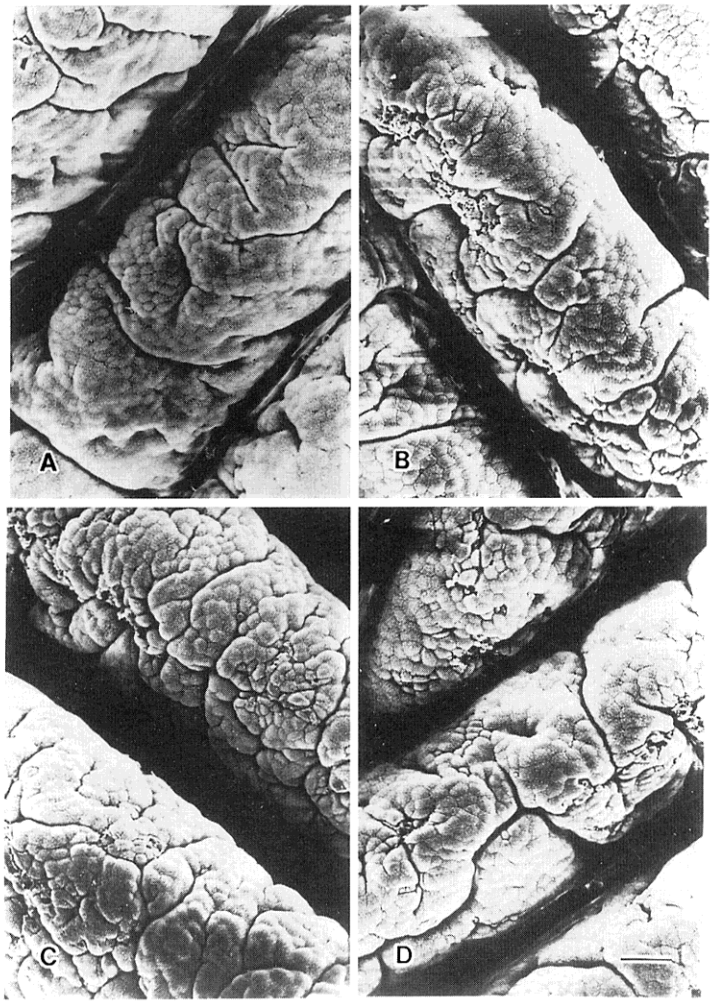

Fig. 3. Jejunal villus surface in chickens given conventional diet (A), 0.1% wood vinegar compound solution (B), 1% charcoal powder diet (C), and 1% charcoal powder with wood vinegar compound diet (D). One can see the activated morphological changes in all experimental substances. Scale bar=23μm, (x 290).

cells covered totally with microvilli. In the case of CWVC group (Fig. 2D), a clear cell outline due to the remarkable cell protuberance was appeared (arrows).

The villus surface of the jejunum was smoother than that of the duodenum in the control group (Fig. 3A), although the cell outline was clear. After giving WVC (Fig. 3B), charcoal (Fig. 3C) and CWVC (Fig. 3D), the cell outline became much clearer than that of control due to each cell protuberance into the lumen, resulting in rough surface.

On the ileal villus tip surface of the control (Fig. 4A) and WVC (Fig. 4B), the cell outline was not clear. However, the clear cell protuberances remained in charcoal (Fig. 3C) and CWVC (Fig. 4D) groups.

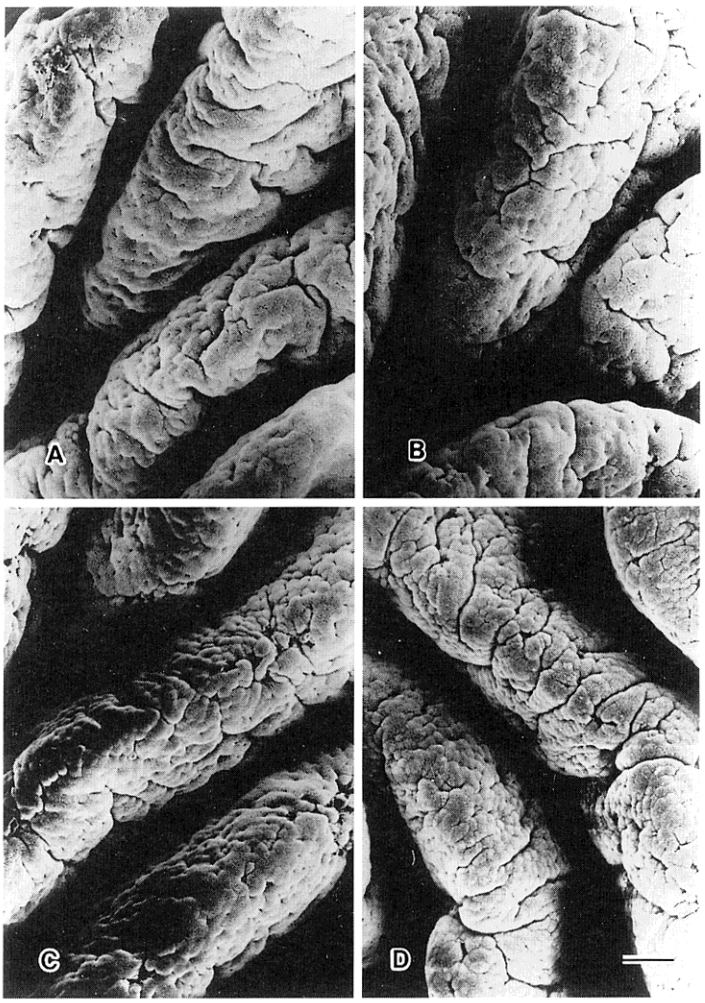

Fig. 4. Ileal villus surface in chickens fed conventional diet (A), 0.1% wood vinegar compound solution (B), 1% charcoal powder diet (C), and 1% charcoal powder with wood vinegar compound diet (D). Cell protuberance (activated morphological changes) remains only in the charcoal powder and charcoal powder with wood vinegar compound diets. Scale bar=23μm, (x 290).

Discussion

In the previous study on the morphological changes of intestinal villi in chickens fed the dietary CWVC diet, we demonstrated that the villus function could be activated at 1% level, inducing the improved feed conversion ratio (Samanya and Yamauchi, 2001). As CWVC is a mixture of WVC and charcoal, the purpose of the present study was to determine which of materials (WVC, charcoal or CWVC) induced such a growth performance and intestinal morphological elevations. Although feed intake and body weight gain were not significantly different among groups, they tended to be

higher in experimental material groups than those of control group. As nutritional composition of fed diets in all groups was almost same, the slightly improved growth performance in the present experimental groups seems to be induced by each dietary non-nutritive sorptive substance. As feed conversion ratio of CWVC group showed the lowest value, the ingested feed might be much more effectively absorbed from the intestinal epithelial cells in this group than that of other groups. This growth performance is confirmed by the morphological results.

Comparison of light microscopic alterations of villi

During the maturation of intestinal epithelium, cells originate by mitosis in the stem-cell zone located in the lower portion of the intestinal crypt and migrate along the villus surface upward to the villus tip within a few days (Imondi and Bird, 1966), where they are extruded into the intestinal lumen within 48 h after formation (Potten, 1998). The values of villus height, cell area and cell mitosis number are known to be increased by the stimulation of the villus absorptive function (Shamoto and Yamauchi, 2000 ; Shamoto *et al.*, 1999 ; Tarachai and Yamauchi, 2000 ; Yamauchi and Tarachai, 2000). In the present study, although these histological values did not change significantly among groups, they tended to show a slight increase after feeding each experimental substance, suggesting that these dietary non-nutritive sorptive substances might stimulate the villus function. As the cell area of the jejunum and ileum were significantly elevated in CWVC group, the CWVC has most effective substance among experimental substances.

Comparison of scanning electron microscopic alterations of villi

Villus tip surface morphological changes seem to be more sensitively reflected by the intestinal function than villus morphological changes observed by light microscopy. On the duodenal villus surface, some cells devoid of any microvilli were observed in the WVC and charcoal groups. In the case of WVC, optimal mycelial growth was observed at 0.07% crude WVC, but it was completely inhibited at the high concentration (Chang *et al.*, 1995). As main components of WVC, 3, 5-dimethylphenol, 2 -methoxyphenol, butanoic acid, 1-pentanol (Yoshimura *et al.*, 1995), acetic acid, propanoic acid and 2, 6-dimethoxy phenol, tetrahydro-2-furylmethanol (Yoshimura and Hayakawa, 1993) were reported. We can not explain at present the reason why microvillus loss was induced after feeding WVC, but the concentration of it would appear to be higher than the optimal level, because the present concentration of WVC was 2 to 3 times that of conventional WVC used by chicken farmers. The growth of bacteria was inhibited at 0.1% concentration of acetic acid in WVC (Entani *et al.*, 1998). The components of WVC might affect the microvillus loss when WVC was used at the high concentration. A further study is being carried out to investigate this direction. In the case of charcoal, the microvillus loss may be induced by sticky physical damage of charcoal powder, because it adhered to villus surface in sampling. However, on the villus tip of the CWVC group, the remarkable cell protuberances appeared without the microvillus loss. As CWVC did not adhere to the villus surface in sampling, the sticky physical feature of charcoal would be changed by adding WVC. The intestinal morphology is well known to be affected by the ingested diets (Langhout

et al., 1999 ; Yasar and Forbes, 1999), and the cell protuberances on the villus tip have been demonstrated to show an activated absorptive function of villi (Shamoto and Yamauchi, 2000 ; Shamoto *et al.*, 1999 ; Tarachai and Yamauchi, 2000). The present clear cell outline due to the remarkable cell protuberance in the CWVC group suggests that duodenal absorptive function might be activated after feeding CWVC diet.

On the jejunal villus surface, the activated absorptive function of villi was found in WVC, charcoal and CWVC groups. In the case of WVC, the reason why the atrophic morphological feature of duodenal villi changed to the activated features in the jejunum may be related to the gradual reduction of WVC concentration with moving caudally. Effects of WVC was demonstrated to be induced by not only crude wood vinegar but by its components such as acetic acid, propanoic acid and butanoic acid (Yoshimura and Hayakawa, 1993 ; Yoshimura *et al.*, 1995). Optimal concentration of WVC enhanced the intestinal absorptive function in rats (Kishi *et al.*, 1999). Diluted components in WVC are thought to be absorbed into the intestinal epithelial cells as nutrients. In the case of charcoal, the finding that adherence of charcoal to villus surface was not found in sampling suggests that the charcoal sticky physical damage reduced in the jejunal part. Charcoal was reported to reduce the effects of toxin in diets by adsorbing it and by preventing its absorption from the intestine (Anjaneyulu *et al.*, 1993). The present dietary charcoal might elevate the jejunal function by adsorbing some kinds of fungal contamination in diets into the charcoal pore, and by preventing their absorption. Consequently, in the case of CWVC, a mixed powder of WVC and charcoal, villus function would be multiplicatively activated by both influences of charcoal and WVC. The present scanning electron microscopic observations suggest that the jejunal absorptive function might be activated in all groups.

In the chickens fed a conventional diet, very little nitrogen absorption was observed beyond the jejunum (Imondi and Bird, 1965), and ingested nutrients were absorbed in the upper part of the intestine (Isshiki *et al.*, 1989). Morphologically, as the ileum did not show a dramatic morphological change even after fasting, the ileum appeared to be relatively inactive in digestive function (Yamauchi *et al.*, 1995 ; Yamauchi *et al.*, 1996). As a characteristic feature, filamentous bacteria adhered to the ileum (Yamauchi *et al.*, 1990), which stimulated the host immune response by elevating IgA in the intestine (Klaasen *et al.*, 1993), which was noted as a possible protective role against infection with *Salmonella enteritidis* in rats (Garland *et al.*, 1982) and enteropathogenic *Escherichia coli* O103 disease in rabbits (Heczko *et al.*, 2000). Recently, phagocytosis and intracellular processing of segmented filamentous bacteria by ileal epithelial cells were also observed, suggesting that phagocytosis could be an important the stimulating effect of these bacteria on the mucosal immune system (Yamauchi and Snel, 2000). These findings might indicate that a main role of the ileum would be the intestinal mucosal immune system. However, in a previous study the activated morphological changes were observed in the ileum of chickens fed 1% dietary CWVC (Samanya and Yamauchi, 2001). Also in the present study, the clear cell protuberances disappeared in WVC group, but remained in charcoal and CWVC groups, suggesting that the ileal absorptive function might be activated in charcoal and CWVC

groups. We think that WVC components were not passed into the ileal part because WVC solution was absorbed in the proximal part of the intestine, these failing to induce the ileal activity. In the case of charcoal and CWVC, components in CWVC remained in the ileum because charcoal is an indigestible (nonabsorbable) substance, inducing the ileal activity. The ileum appears to be richer in intestinal microflora population than the duodenum and jejunum (Gordon and Pesti, 1971 ; King and Toskes, 1979 ; Smith, 1965). In the ileum, a low population of few species of bacteria may produce the acceleration of the cell migration without enhancement of cell mitotic activity (Ishikawa *et al.*, 1986). The acetic acids included in WVC (Yoshimura and Hayakawa, 1993 ; Yoshimura *et al.*, 1995) were reported to control the balance of intestinal microflora and pathogen (Pinheiro *et al.*, 1968 ; Sorrells and Speck, 1970). A consideration of the present result and the findings of similar studies in the literature leads to the general conclusion that the ileum also participates in absorptive function when it contains intestinal contents including absorbable nutrients, inducing the present slight elevation of chicken growth performance.

In conclusion, among WVC, charcoal, and CWVC substances, the CWVC was most effective substance to activate the intestinal absorptive function, and the functional activation of whole intestine including the ileum due to CWVC may induce a slight elevation of chicken growth performance.

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