Histology of intestinal villi and epithelial cells in chickens fed low-crude protein or low-crude fat diets

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Abstract
To establish the intestinal histological alterations in chickens fed low-crude protein (CP) or low-crude fat (EE) in long-term isocaloric diets, 60 birds were allotted to three treatments, each with five replicates of four chickens. They were fed the control, low-CP or low-EE diet from the age of 9 to 16 weeks. The chickens receiving the low-CP diet showed a clear reduction in performance and carcass characteristics like breast and wings. However, these adverse effects did not occur in the chickens fed the low-EE diet. The villus height in the duodenum and ileum were lower (P<0.05) in the low-CP group. The duodenal villus area decreased (P<0.05) in both the low-CP and low-EE groups, whereas the ileum showed a lower value (P<0.05) in only the low-CP group. The cell area of the duodenum and jejunum displayed decreasing values (P<0.05) in the low-CP and low-EE groups. As regards the mitotic numbers, the jejunum and ileum showed a decrease (P<0.05) in the low-CP group. On the villus tip surface in the duodenum of the control and low-EE groups, clear protuberant cells and cell clusters with a great number of epithelial cells were found. These cell clusters degraded to faintly dome-shaped cells in the low-CP group, resulting in a lower level of morphological changes than in the other two groups. The epithelial cells on the jejunal and ileal tip surfaces did not show a specific change in the treatment groups.

Introduction
Protein and fat are absolutely essential for animal growth, reproduction and maintenance. Lowering the crude protein (CP) or crude fat (EE) content of poultry diets may have an effect not only on growth rate and reproduction but also on the ontogeny and morphology of the gastrointestinal tract. Reports of gross anatomical changes of the gut owing to the type of diet (Langhout et al., 1999; Yasar and Forbes, 1999) and striking alterations of villus shape in rats fed a no-fibre diet (Tasman-Jones et al., 1982) have been published. The small intestine, especially the intestinal villi and absorptive epithelial cells, play significant roles in the final phase of nutrient digestion and assimilation. In starved-refed trials, the values of villus height, cell area and cell mitosis were low after starving but clearly increased when the animals were refed the formula diets. The epithelial cells on the villus tip also showed a smooth surface after starving but changed to a rough surface after refeeding (Yamauchi, 2002). These results seem to suggest that the morphological changes of the intestinal villi are dependent on the presence of digested nutrients in the small intestinal lumen. In chickens refed single nutrients of protein, carbohydrate or fat cannot recover to the levels of the formula diet in light microscopic parameters such as villus height, villus areas, cell area and cell mitosis; therefore, it is likely that a single-nutrient diet cannot induce histological recovery of the intestinal villi (Maneewan and Yamauchi, 2005). Similarly, among the semi- purified protein-free, fat-free and fibre-free pellet diets, the protein-free diet was the slowest in promoting histological recovery, suggesting that protein is the most important factor in recovery after feed withdrawal (Maneewan and Yamauchi, 2004). All of these experiments have used the strategy of starving and refeding with diets including those composed of several nutrients to analyse the intestinal morphology. However, a long-term feeding strategy seems to be an interesting method of examining intestinal histological changes. Recently, although the assessment of the histology of intestinal villi and epithelial cells using long-term feeding of low-CP diets has been reported (Buwjoom et al., 2010), no information regarding the effects of dietary EE on the histology of the small intestine has been published to date.

The aim of our study was to establish the histological alterations of the intestinal villi and absorptive cells in chickens fed low-CP or low-EE in long-term isocaloric diets, and to determine between CP and EE which nutrient is more important for intestinal morphology.
tered by bleeding the left jugular vein, and their feathers plucked. Head, viscera and shanks were removed. The carcass was left for one hour to remove excess water and then weighed. Breast, wings, drumsticks, thighs, heart, liver and the proventriculus and gizzard were obtained and weighed individually.

For the gross anatomical examination of the small intestine, the entire small intestine from the gizzard to the large intestine was removed. The small intestine consists of three segments. The first segment, the duodenum, extends from the gizzard to the pancreas and forms a loop surrounding most of the pancreas. The second segment is the jejunum, extending from the distal portion of the duodenal loop to Meckel’s diverticulum. The third segment, the ileum, extends from Meckel’s diverticulum to the ileo-caecal junction. Intestinal length and weight were measured in each segment. All data from these measurements were calculated and expressed as g/100g BW and intestinal length was express as cm/100g BW.

Tissue sampling procedure for intestinal histology

At 16 weeks of age, five birds per group were euthanised with diethyl ether, and the whole small intestine was removed immediately and put into a mixture of 3% glutaraldehyde and 4% paraformaldehyde fixative solution in 0.1 mol/L sodium cacodylate buffer (pH 7.4). The specimens were cut from three segments (the duodenum, jejunum and ileum).

Light microscopic examination

The intestinal segments were kept in Bouin’s solution and dehydrated in a graded series of alcohols. Finally, each specimen was embedded in paraffin wax and cut at 4 µm. Sections were fixed on glass slides and stained with haematoxylin-eosin. For measurement of villus height and area, two villi were randomly selected from each cross-section. The villus length from tip to base, excluding the intestinal crypt, was measured. The width of the villus was measured at the basal and apical parts. A total of 16 villus heights from eight cross-sections were measured and regarded as the mean for each bird. The villus area was calculated from the villus height, basal width and apical width (Iji et al., 2001). The 16 calculations of villus area were expressed as the mean for each bird. For cell area measurement, a single epithelial cell area on a 4 µm cross-section was measured at the middle part of the villus and the cell nuclei within this area were counted. Ultimately, the area of the cell layer was divided by the number of cell nuclei.

These eight calculated values from four different sections were regarded as the mean cell area for each bird. For the cell mitosis count, mitotic cells having homogenous, strongly stained basophilic nuclei were considered mitotic cells. Cell mitosis numbers were counted in all intestinal crypts from four sections, and an average of these values was expressed as the mean cell mitosis number for each bird. All light microscopic data were measured using an image analyzer (Nikon Cosmozone Ltd., Tokyo, Japan).

Scanning electron microscopic examination

Each segment was opened lengthwise and washed with 0.1 M phosphate buffered saline (pH 7.4). The specimens were fixed in a mixture of 3% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) at room temperature for one hour. Afterwards, each sample was cut into a 5×6 mm square and fixed for a further one hour. The pieces were rinsed with 0.1 mol/L sodium cacodylate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide in ice-cold buffer for two hours. All specimens were kept in 70% alcohol. After freeze-drying, the dried specimens were coated with platinum (RMC-Eiko RE Vacuum Coater, Eiko Engineering Co. Ltd., Tokyo, Japan) and viewed in a Hitachi S-4300SEM scanning electron microscope (Hitachi Co., Ltd., Tokyo, Japan). Morphological changes of the absorptive epithelial cells on the villus tip surface were compared among the groups.

Statistical analysis

Data on growth performance carcass yield, intestinal weight and length, and light microscopic examination (villus height, villus area, cell area and cell mitosis number) were expressed as the mean ± standard error for each group. Differences between groups were determined using the one-way ANOVA test (Scheffe’s multiple comparison). Differences were considered significant at P<0.05.

Table 1. Chemical composition of the experimental diets (% w/w dry basis).

| Item                      | Control | Low-CP | Low-EE |
|--------------------------|---------|--------|--------|
| Crude protein            | 18.10   | 9.40   | 18.10  |
| Ether extract            | 4.90    | 3.90   | 2.90   |
| Crude fibre              | 3.80    | 3.70   | 3.30   |
| Ash                      | 6.00    | 5.90   | 6.10   |
| Metabolisable energy, kcal/kg | 2.852 | 2.853 | 2.854 |
| Calcium                  | 1.20    | 1.20   | 1.20   |
| Phosphorus (total)       | 0.66    | 0.72   | 0.67   |
| Phosphorus (available)   | 0.38    | 0.38   | 0.38   |
| Lysine                   | 0.93    | 0.47   | 0.93   |
| Methionine               | 0.32    | 0.16   | 0.32   |
| Methionine + cystine     | 0.61    | 0.54   | 0.62   |
| Tryptophan               | 0.22    | 0.11   | 0.20   |
| Threonine                | 0.70    | 0.32   | 0.69   |

CP, crude protein; EE, crude fat.

Figure 1. Light microscopic examinations of the duodenum, jejunum and ileum in chickens, 9-16 weeks old, fed control, low-crate protein or low-crate fat diets. * Means within each group with different letter designations differ (P<0.05).

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analysed with one-way analysis of variance (ANOVA) using the SPSS statistical software package (version 10.0 for windows, SPSS, Inc., Chicago, IL, USA). Differences among the treatments were analysed by Duncan’s multiple range test. P<0.05 were considered significant.

Results

Growth performance

Compared with the control group, weight gain and feed efficiency decreased (P<0.05) in the low-CP group, while no significant differences was observed in the low-EE group (Table 2). However, there were no significant differences in feed intake among the groups.

Carcass yield and intestinal weight and length

There were no significant differences in dressing, drumsticks, thighs, heart, liver, and proventriculus and gizzard among the treatments (Table 2). However, the value of the breast decreased (P<0.05) in the low-CP group, and the weight of the wings was lower (P<0.05) in both the low-CP and low-EE groups than in the control group. The low-CP group showed a heavier jejunum (P<0.05) than the other groups. However, no significant differences were presented in duodenal and ileal weights. Both the low-CP and low-EE groups had a significantly longer duodenum than the control. Although the jejunal length of the low-CP group was not significantly different from that of the control group, it was increased (P<0.05) compared to that of the low-EE group. There was no significant difference in the ileal length among the treatments.

Intestinal villus height, villus area, cell area and cell mitosis number

Compared with the control, the villus heights of duodenum and ileal were lower (P<0.05) in the low-CP group. Duodenal villus area decreased (P<0.05) in both the low-CP and low-EE groups, whereas the ileal part showed a lower value (P<0.05) in only the low-CP group. The cell areas of the duodenum and jejunum displayed a decreasing value (P<0.05) in the low-CP and low-EE groups. As regards the mitotic number in the crypt cells, the jejunum and ileum showed a decrease (P<0.05) in the low-CP group (Figure 1).

Epithelial cells on the villus tip

On the villus tip surface in the duodenum of the control and low-EE groups (Figures 2A and C), clear protuberant cells and cell clusters with a great number of epithelial cells were found. Conversely, these cell clusters degraded to faintly dome-shaped cells in the low-CP group (Figure 2B), resulting in lower levels of morphological changes than in the other two groups. However, epithelial cells on the jejunal and ileal tips surfaces did not show a specific change among the treatment groups (Figures 3 and 4).

| Item                      | Control          | Low-CP           | Low-EE           |
|---------------------------|------------------|------------------|------------------|
| Weight gain, kg/bird      | 0.93±0.04ab      | 0.69±0.05bc      | 0.90±0.04ab      |
| Feed intake, kg/bird      | 4.63±0.12        | 4.73±0.08        | 4.51±0.11        |
| Feed efficiency           | 0.20±0.005a      | 0.15±0.009b      | 0.20±0.004a      |
| Carcass yield, g/100 g BW |                  |                  |                  |
| Dressing°                 | 76.92±1.70       | 73.58±1.94       | 74.80±1.16       |
| Breast                    | 10.72±0.33ab     | 8.66±0.54bc      | 10.12±0.25ab     |
| Wings                     | 10.79±0.18ab     | 9.86±0.23bc      | 9.40±0.37ab      |
| Drum-sticks               | 11.68±0.21       | 11.39±0.58       | 10.98±0.41       |
| Thighs                    | 14.83±0.37       | 13.34±0.56       | 14.46±0.48       |
| Heart                     | 0.53±0.04        | 0.54±0.06        | 0.53±0.04        |
| Liver                     | 1.73±0.11        | 1.73±0.07        | 1.48±0.05        |
| Proventriculus and gizzard| 1.73±0.08        | 1.96±0.13        | 1.66±0.09        |
| Duodenum                  | 0.31±0.05        | 0.36±0.03        | 0.34±0.02        |
| Jejunum                   | 0.52±0.04ab      | 0.64±0.03ab      | 0.49±0.03ab      |
| Ileum                     | 0.42±0.05        | 0.48±0.01        | 0.43±0.02        |
| Intestinal weight, g/100g BW |                |                  |                  |
| Duodenum                  | 0.84±0.04ab      | 1.10±0.05ab      | 1.04±0.04ab      |
| Jejunum                   | 2.33±0.10ab      | 2.56±0.14ab      | 2.12±0.05ab      |
| Ileum                     | 2.42±0.12        | 2.59±0.08        | 2.40±0.02        |

Means within each grouping with different letter designations differ (P<0.05); °eviscerated carcass with neck and feet; #intestinal contents removed.

Figure 2. Villus tip surface in the duodenum of chickens, 9-16 weeks old, fed the control (A), low-crude protein (B) or low-crude fat (C) diets. Low-CP group showed a lower level of morphological changes than the other two groups. Scale bar = 50 µm, magnification: x 600.
Discussion

All experimental diets had a lower ME (kcal/kg) and EE (%) when compared with the conventional broiler diets. In Japan, broiler chickens which can reach a BW of 3.0-3.5 kg by 7 weeks of age clearly have much higher weight gain than the Sanuki Cochin breed, which may take 20 weeks to reach 2.5-3.0 kg BW. These nutritional differences relate to growth rate of each strain; therefore, slow-growing strains have lower nutritional and energy requirements than fast-growing strains. However, our current study has established the effects of low-CP or low-EE content in isocaloric diets on the intestinal histology of Sanuki Cochin male chickens. To our knowledge, this is the first report demonstrating the effect of lower than 48% CP and 41% EE content in diets on the histological properties of the intestinal villus and absorptive epithelial cell. During long-term feeding of a low-CP diet, chickens have shown a significant reduction (P<0.05) in weight gain and feed efficiency, but these adverse effects did not occur when they were fed a low-EE diet. Furthermore, carcass characteristics like breast and wing yield decreased significantly (P<0.05) in the low-CP group. Additionally, many research studies have reported that growth rate and carcass characteristics tend to be inferior in broiler chickens (Fancher and Jensen, 1989a,b,c; Aletor et al., 2000; Bregendahl et al., 2002; Sterling et al., 2005; Waldroup et al., 2005) and pigs (Kerr et al., 1995; Tuitoek et al., 1997; Knowles et al., 1998) fed diets in which dietary CP content is lowered by more than three to four percentage points. A low-CP diet causes a diminution in the rate of protein synthesis in most body tissues, especially in the intestine (Wykes et al., 1996). This implies that a reduction in dietary CP would also affect the intestinal morphology. Gross anatomically, an increase in the length and weight of the jejunum and ileum was reported in malnourished rats (Nieto et al., 2000). A hypo-nutrient diet can also induce an increase in intestinal length (Olkowski et al., 2005). The increased weight and length (P<0.05) of the duodenum and jejunum of the low-CP group might be a result of a compensatory mechanism to increase the absorptive capacity in an attempt to assimilate any nutritional benefit from a hypo-protein diet.

Numerous histological studies have revealed that the intestinal villi and cells in piglets (Mekbungwan et al., 2003), ducks (Khambualai et al., 2009; Ruttanavut et al., 2009) and chickens (Incharoen and Yamauchi, 2009; Incharoen et al., 2009) are well recognized to be affected by dietary components. Our current histological findings show that duodenal and ileal villus height were decreased (P<0.05) in the low-CP group, and duodenal villus area tended to be decreased (P<0.05) in both the low-CP and low-EE groups, while the ileum showed a lower value (P<0.05) in only the low-CP group. Similarly, previous reports in chickens refed unbalanced nutrient compositions have demonstrated that intestinal villi were shorter and narrower than...
in chickens refed a nutritionally well-balanced diet (Maneewan and Yamauchi, 2003, 2004, 2005). Decreasing values for villus characteristics corresponded with reductions in the villus surface area, enzyme activities such as mucosal lactase and sucrase (Park et al., 1998), lactase and alkaline phosphatase (Zijlstra et al., 1997), total lactase phosphohydrolase and mucosal protein concentration (Dudley et al., 1998), and alkaline phosphatase and disaccharidase (López-Pedrosa et al., 1998). In villus cellular morphology, our finding was that the cell areas of the duodenum and jejunum displayed a decreasing value (P<0.05) in the low-CP and low-EE groups. Likewise, the area of the epithelial cells also tended to be decreased in chickens refed a semi-purified protein- and fat-free pellet diet, compared with the semi-purified well-balanced pellet diet group (Maneewan and Yamauchi, 2004).

Intestinal epithelial cells are derived from cells situated in the intestinal crypt base (Cheng and Leblond, 1974; Potten, 1998; Potten and Grant, 1998; Wilson et al., 1998). Cells proliferate by mitosis in the crypts, differentiating as they migrate upward to each villus and reach the villus tips (Imondi and Bird, 1966), where they are shed into the intestinal lumen within 48 hours after birth (Potten, 1998). Epithelial cell proliferations were reduced as a result of decreased intake of energy (Fleming et al., 1994) and nutrients (Tarachai and Yamauchi, 2000), while fat exerted a strong stimulatory effect for intestinal mucosal regrowth (Buts et al., 1990). In this report, a low-CP diet reduced the number of mitosis cells (P<0.05) in the jejenum and ileum. Morphological analysis of mitotic cell numbers in the crypt indicated that CP might be the most important nutrient among macronutrients for mitotic activity. Furthermore, cells at the top of the villus seem to be sensitively affected by nutritional content. Epithelial cells showed a hypertrophied morphology in chickens (Maneewan and Yamauchi, 2004) and pigs (Mekbungwan et al., 2003) fed a high CP diet. High protein–low energy diets induced many more protuberated cells than low protein–high energy diets (Yamauchi et al., 1993). Although epithelial cells on the jejunal and ileal villus tip surfaces did not show a specific change among the treatment groups, the duodenal villi displayed an inferior level of morphological changes than those of the control and low-EE groups. These observations suggest that the activity of the duodenal epithelial cells might be atrophied with decreasing CP content, resulting in the faint cells in the low-CP group.

Conclusions

During long-term feeding of low-CP or low-EE diets, chickens receiving a low-CP diet showed a clear reduction not only in performance and carcass characteristics, such as breasts and wings, but also in the histology of the intestinal villi and duodenal epithelial cells. However, these adverse effects did not occur in chickens fed a low-EE diet. It seems that CP is the most important among the dietary macronutrients for the development of the morphological features of the villi and epithelial cells.

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