Title: Influences of protein ingestion on glucagon-like peptide (GLP)-1 -immunoreactive endocrine cells in the chicken ileum

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Running Head: INFLUENCE OF PROTEIN INGESION ON CHICKEN L CELL

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ABSTRACT

Influences of a specific dietary nutrient on glucagon-like peptide (GLP)-1-containing cells in the chicken intestine are not yet clear. Significance of dietary protein level on GLP-1-containing cells in the chicken ileum was investigated. Chickens fed control or experimental diets of varying protein level were examined using immunohistochemical and morphometrical techniques. We show that the protein ingestion had an impact on the activities of GLP-1-immunoreactive cells in the chicken ileum. Weight gains declined with decreasing dietary crude protein (CP) levels, but no significant differences were detected in the daily feed intake and villous height. GLP-1-immunoreactive cells with a round or oval shape were frequently observed in the lower CP level groups (4.5% and 0%). Frequencies of occurrence of GLP-1-immunoreactive cells were 41.1 ± 4.1, 38.5 ± 4, 34.8 ± 3.1 and 34.3 ± 3.7 (cells/mm^2, mean ± SD) for dietary CP level of 18%, 9%, 4.5% and 0 % groups, respectively and significant differences were recognized between the control and lower CP level groups ($P<0.05$). Multiple regression analysis indicated a significant correlation between the daily protein intake and frequencies of occurrence of GLP-1-immunoreactive cells. The protein ingestion is one of the signals that influence GLP-1-containing cells in the chicken small intestine.

Key words*: chicken, glucagon-like peptide-1, immunohistochemistry, intestine, protein ingestion.*

INTRODUCTION

Glucagon-like peptide-1 (GLP-1) consists of 36-amino acids and derived from specific post-translational proteolytic cleavage and other enzymatic modifications of proglucagon. This meal-induced gut hormone (Elliott *et al.* 1993; Balkan 2000) is secreted from L cells in the mammalian intestine (Drucker 2006) and has many important physiological actions such as the stimulation of glucose-dependent insulin biosynthesis and release, the inhibition of glucagon secretion from pancreatic A cells (Fridolf *et al.* 1991; Nauck 1998; Drucker 2006), reduction of food intake (Drucker 2006), deceleration of gastric emptying (Nauck 1998; Schirra *et al.* 2006) and suppression of intestinal motility (Tolessa *et al*. 1998a, b). Our previous immunohistochemical and morphometrical studies in the chicken intestine showed that the immunoreactive cells against GLP-1 antiserum are mainly distributed in the whole jejunum and ileum, and rarely found in the ascending duodenum, but not observed in other intestinal regions (Hiramatsu *et al.* 2003; 2005). These findings suggest that GLP-1 plays an important role in the regulation of activities of the chicken small intestine.

GLP-1-secreting L cells are scattered in the villous epithelium and crypts of the chicken small intestine. L cells in the chicken small intestine are covered apically with microvilli, open-typed, and contained many secretory granules in their perikarya (Nishimura *et al*. 2013). These ultrastructural features indicate that the secretion of GLP-1 is induced by the ingested feed in the chicken intestine. In fact, the restricted feeding influences the frequency of occurrence of GLP-1-immunoreactive cells in the chicken small intestine (Monir *et al.* 2013). However, whether a specific dietary nutrient influences the secretion of GLP-1 from L cells in the chicken intestine is not yet clear. The influences of the protein ingestion on GLP-1 secretion are particularly obscure even in the mammals.

In the present study, we aimed to clarify the influence of dietary protein level on

GLP-1-containing endocrine cells in the chicken ileum using immunohistochemical and morphometrical methods. The significance of the protein ingestion on the intestinal L cells is discussed.

MATERIALS AND METHODS

Experimental birds

Male White Leghorn chicks at one day of age (n=20) were commercially obtained from Komatsu Shukeijyo (Matsumoto, Nagano, Japan) and reared in our laboratory. The birds were given a commercial diet containing 17% crude protein (CP) and water *ad libitum*, and housed under the continuous lighting for 24 h up to 6 weeks of age. The birds were then fed the control diet described below under the controlled light condition (12 h light: 12 h darkness) for 3 days to adapt them to the experimental diet.

Experimental feeding

Chickens (average body weight 389.9 g) were randomly divided into four groups (control group: CP 18% , CP 9%, CP 4.5% and CP 0%) of five birds each based on average body weight and fed in separate cages with the experimental diet shown in Table 1 under the controlled light condition (12 h light: 12 h darkness). The well-balanced experimental diets contained the same protein source (isolated soybean protein) as that of the control group. To keep the energy density constant (ME= 2,847-2,850 kcal/kg DM), the inclusion of cornstarch was increased in diets of the experimental groups. The 18% of CP and 2,850 kcal/kg of ME were identical to the CP and ME requirements recommended by Japanese Feeding Standard for Poultry (NARO 2011). During the 7-day experimental feeding period, the daily feed intake (g/day) and body weight (g) of each bird were recorded each day at the same time (10.00 hour) and in the same manner. The average value of the daily protein intake (g/day) of each chicken during the experimental period was calculated from the

average value of the daily feed intake and CP level (%) of the experimental diet. Chickens were treated in accordance with the "Guideline for Regulation of Animal Experimentation (1997)" of the Faculty of Agriculture, Shinshu University.

Insert here Table1.

Tissue samples

At the end of the experimental feeding period, chickens were sacrificed by decapitation. Because frequencies of occurrence of GLP-1-immunoreactive cells were highest in distal ileum and low in jejunum of the chicken small intestine (Hiramatsu et al. 2003; 2005), distal ileum (at the middle level of ceca), approximately 2 cm long, was immediately dissected from each bird after examining the gross anatomy of the abdomen. Contents of tissue samples were washed with 0.75% sodium chloride solution and immersed in Bouin's fluid at room temperature for 24 h.

Immunohistochemistry

Tissue samples were embedded in paraffin wax according to standard procedures. Sections were cut at 5 μm thickness and expression of GLP-1 was assessed by immunohistochemical techniques. The streptavidin-biotin method (Guesdon et al., 1979) was used to detect GLP-1-immunoreactive cells according to the procedures previously described by Hiramatsu and Ohshima (1995). Rabbit antiserum against synthetic GLP-1(1-19) conjugated to bovine serum albumin (Affiniti Research Products, UK, No.GA1176, diluted to 1:2000) was used as the primary antibody. This antiserum shows no cross-reactivity with other proglucagon-derived peptides such as glucagon and GLP-2 (Tachibana *et al*. 2005). Primary antibodies against GLP-1 described above was preabsorbed with chicken/common turkey GLP-1(7-36) amide (10^{-6}M/ml, H-5824; Bachem, Bubendorf, Switzerland) at 4°C for 24 h and then used for confirming the specificity. Preabsorbed GLP-1 antibody showed the negative reaction. Negative control sections were incubated with the normal rabbit serum

instead of the specific primary antibody or in the absence of the primary antibody. The negative controls showed negative immunoreactivity.

Morphometry

The villous height (μm) of 10 well-oriented villi was measured in each chicken. Fifty villi in total were measured per group and the average value was calculated for each group. To evaluate the frequency of occurrence of GLP-1-immunoreactive cells in each group, the immunoreactive cells with clearly identifiable nuclei were counted, and the area of the mucosal layer was measured. The cell number per area of the mucosal layer (cells/mm^2) was then calculated. These measurement and quantification were performed using a computerized image analyzing system (KS400; Zeiss, Göttingen, Germany). Twenty areas were measured in each bird. One hundred areas in total were measured from 5 chickens in each group.

Statistic analysis

Statistical analysis was performed to assess the differences in the villous height and the frequency of GLP-1-immunoreactive cells among the four groups using Tukey's method (Yanai 2011). Multiple regression analysis using the General Linear Model Procedures (SAS/STAT) was performed between the daily protein intake and the frequency of occurrence of GLP-1-immunoreactive cells.

RESULTS

Influences of dietary protein level on body weight gain, feed intake and villous height and histology

Measurements were obtained from chickens divided into four experimental groups based on dietary protein levels over a 7-day experimental feeding period. Body weight gains were declined correspondingly with a decrease in dietary CP levels, and

was negatively balanced in the CP 0% group. There was, however, no significant difference in the daily feed intake among the four experimental groups (Table 2). The villous height in the CP 0% and CP 4.5% groups tended to be lower than that of the control group, but the differences were not significant. The form of villous tips in the CP 0% and CP 4.5% groups tended to be dull compared with those of the control group (Fig. 1a, b).

Insert here Table2.

Influences of dietary protein level on GLP-1-immunoreactive cells

A large number of endocrine cells immunoreactive for GLP-1 antiserum were observed scattering in the ileal epithelium of all groups (Fig. 1a, b). Most of the cells had a pyramidal or spindle-like shape in the villous epithelium (Fig. 1c) and a comma-like shape in crypts (Fig. 1 d, f) and were in contact with the intestinal lumen through their apical cytoplasmic process. GLP-1-immunoreactive cells with a round or oval shape were observed in lower CP level groups (4.5% and 0%), with higher numbers in the CP 0% group (Fig. 1e, arrows). In the control group, GLP-1-immunoreactive cells were primarily dispersed in crypts and the epithelium of middle and lower parts of intestinal villi (Fig. 1a). In CP 0% and CP 4.5% groups, however, these cells were more commonly observed in crypts and less frequent in the epithelium of the middle part of intestinal villi (Fig. 1b).

Insert here Fig1.

In the control group, the frequency of occurrence of GLP-1-immunoreactive cells was 41.1 ± 4.1 (cell number per mucosal area: cells/mm^2, mean ± SD). In the CP 9%, CP 4.5% and CP 0% groups, these values were 38.5 ± 4, 34.8 ± 3.1 and 34.3 ± 3.7 cells/mm^2, respectively. There were significant differences in the frequency of occurrence of GLP-1-immunoreactive cells between the control group and lower CP level groups ($P<0.05$). The frequencies of occurrence of GLP-1-immunoreactive cells were decreased with the steady decline of the dietary CP level (Fig. 2).

The daily protein intake (average of the daily feed intake × CP% / 100) was

calculated for each bird in each group (Table 2). Multiple regression analysis using the General Linear Model Procedures indicated a significant correlation between the daily protein intake (X) and the frequency of occurrence of GLP-1-immunoreactive cells (Y). The regression equation was as follow: $Y = 25.92 + 1.87X$, $P<0.0001$ (Fig. 3).

Insert here Figs2&3.

DISCUSSIONS

In the present study, chickens were given four experimental diets with varying dietary CP levels from 0 to 18%. As shown in Table 2, although there were no significant differences in daily feed intakes among all experimental treatments, body weight gain was declined by decreasing of dietary CP levels and was negatively balanced in the CP 0% group. The lowered body weight gain in low CP groups would be due to the decrease in daily CP intakes.

The secretion of GLP-1 from intestinal L cells is stimulated in response to ingestion of mixed meals or macronutrients in mammals (Elliott *et al.* 1993; Balkan 2000). However, it has not yet been clear if a specific nutrient could stimulate the GLP-1 secretion from L cells in the chicken intestine. The present study demonstrated that the protein ingestion had an impact on GLP-1-immunoreactive cells in the chicken ileum. Frequencies of occurrence of GLP-1-immunoreactive cells were decreased with the steady decline of the dietary CP level and significant differences were recognized between the control group and lower CP level groups (4.5% and 0%). A positive correlation was also observed between the frequency of occurrence of GLP-1-immunoreactive cells and the daily protein intake. Moreover, GLP-1-immunoreactive cells with a round or oval shape were frequently observed in the CP 0% group. Such a kind of endocrine cells was observed in the intestinal epithelium of the fasted chickens. They showed degenerating features, e.g. an appearance of vacuoles in the perikaryon and lobulated nuclei, at the ultrastructural

level (our unpublished data). These morphological results indicate that the amount of ingested protein is one of the important factors that influence the activities of L cells in the chicken small intestine. L cells are open-typed cells in contact with intestinal lumen by their cytoplasmic processes covered apically with microvilli. These cells function as the chemosensors that monitor nutrients in the ingested food (Breer *et al*. 2012). We recently demonstrated that L cells in the chicken small intestine were covered apically with microvilli (Nishimura *et al*. 2013) and revealed that restricted feeding influenced their frequency of occurrence (Monir *et al*. 2013) Thus, L cells might monitor the content of ingested feed and secrete GLP-1 to conduct physiological phenomena in the chicken.

Many studies have shown that specific nutrients affect GLP-1 secretion from intestinal L cells in mammals including human. Carbohydrates are one of the strong stimuli for GLP-1 release from intestinal L cells (Elliott *et al.* 1993; Herrmann *et al*. 1995). Some studies indicated that glucose ingestion influenced GLP-1 secretion in healthy humans (Qualmann *et al*. 1995; Salehi *et al.* 2008). Herrmann *et al*. (1995) demonstrated that the oral administration of glucose induced GLP-1 release, but intravenous glucose had no effect. Yoder *et al*. (2010) showed the dose-dependent relationship between GLP-1 secretion and amounts of dietary carbohydrate in lean rats. Fat also induces GLP-1 secretion, as fat ingestion was found to increase the plasma level of GLP-1 in healthy men without affecting glucose level (Carr *et al*. 2008). Yoder *et al.* (2009) showed that lipid stimulated GLP-1 secretion in a dose-dependent manner. However, the increase of GLP-1 secretion by fat ingestion is delayed compared with carbohydrates (Elliot *et al*. 1993). These reports confirm carbohydrate and fat are stimuli for GLP-1 secretion from L cells in the mammalian intestine.

Several studies have shown that protein ingestion could stimulate GLP-1 release from intestinal L cells. High protein diets induced the increase of the plasma

concentration of GLP-1 in healthy humans (Blom *et al.* 2006; Lejeune *et al.* 2006). Intraduodenal infusion of protein also increased the plasma concentration of GLP-1 in lean, healthy men (Ryan *et al*. 2012). GLP-1 outputs, however, did not respond in a dose-dependent manner to increasing amounts of dietary protein in lean rats (Yoder *et al*. 2010). Effects of ingested protein on GLP-1 secretion differ according to their sources because of their different amino acid profile.

Our findings presented here indicate that L cells are responsive to protein ingestion in chickens. This response in chicken may be dependent on amounts of protein intake, because the frequency of occurrence of GLP-1-immunoreactive cells was significantly decreased in parallel with the reduction of the daily protein intake. GLP-1-immunoreactive cells in lower CP level groups showed different localization and morphology compared with those in the control group. These data lead to the hypothesis that ingested protein influences the proliferation of L cells in chicken ileum. Yusta *et al.* (2000) showed that L cells with GLP-1 immunoreactivity in the human small intestine coexpress GLP-2-receptor. GLP-2 is also released from L cells in response to ingestion of meals and promotes crypt cell proliferation. In the chicken small intestine, GLP-1 and GLP-2 are costored in the same secretory granules of L cells (Nishimura *et al*. 2013). Reduction of the daily protein intake induces a decreased secretion of GLP-1 and GLP-2 as the proliferation of L cells might be suppressed and the frequency of occurrence of GLP-1-immunoreactive cells was cut downed. Until now, there has been no documented evidence showing expression of GLP-2 receptor in L cells of the chicken small intestine. Systematic biochemical studies are necessary to resolve this crucial issue.

In the present study, we increased the inclusion of cornstarch in the experimental groups to keep the energy level constant (ME= 2,847-2,850 kcal/kg DM). Carbohydrates are one of the strong stimuli for GLP-1 release from intestinal L cells of mammals (Elliott *et al.* 1993; Herrmann *et al*. 1995). If increased amount of

cornstarch have any stimulative effects on L cells of the chicken intestine, morphological changes could be obvious in the lower CP groups. But such effects were not observed on L cells even in the 0% CP groups. That is to say, cornstarch may induce no stimulative effect on L cells of the chicken intestine.

The mechanism of GLP-1 secretion is very complex, as many circulating or locally derived peptide-hormones are involved in this process. Glucose-dependent insulinotropic polypeptide (GIP) is one of such hormones. GIP is secreted from K cells in response to food ingestion and functions as the incretin hormone. In the mammalian intestine, K and L cells are localized in the proximal and distal small intestine, respectively (Buchan *et al*. 1978; Bunnett & Harrison 1986; Damholt *et al.* 1999). In humans, GLP-1 exhibits a biphasic secretory profile with early and late phases following meal ingestion (Herrmann *et al*. 1995). Because GIP stimulates GLP-1 secretion from L cells in mammals (Roberge & Brubaker 1993; Damholt *et al.* 1998), early-phase GLP-1 secretion is induced by GIP and late-phase by nutrients in the intestinal lumen. Recently, GIP-immunoreactive (K) cells were reported in the villous epithelium and crypts of chicken small intestine (Pirone *et al.* 2011). GIP shows a high response after protein ingestion in healthy men (Carr *et al*. 2008). It is likely that GLP-1 secretion is regulated by GIP in the chicken small intestine.

Conclusion

The present data are relevant to the conclusion that the protein ingestion makes an impact on L cells in the chicken small intestine since the frequencies of occurrence of GLP-1-immunoreactive cells were decreased with the steady decline of the dietary CP level and showed a significant correlation with the amount of daily protein intake.

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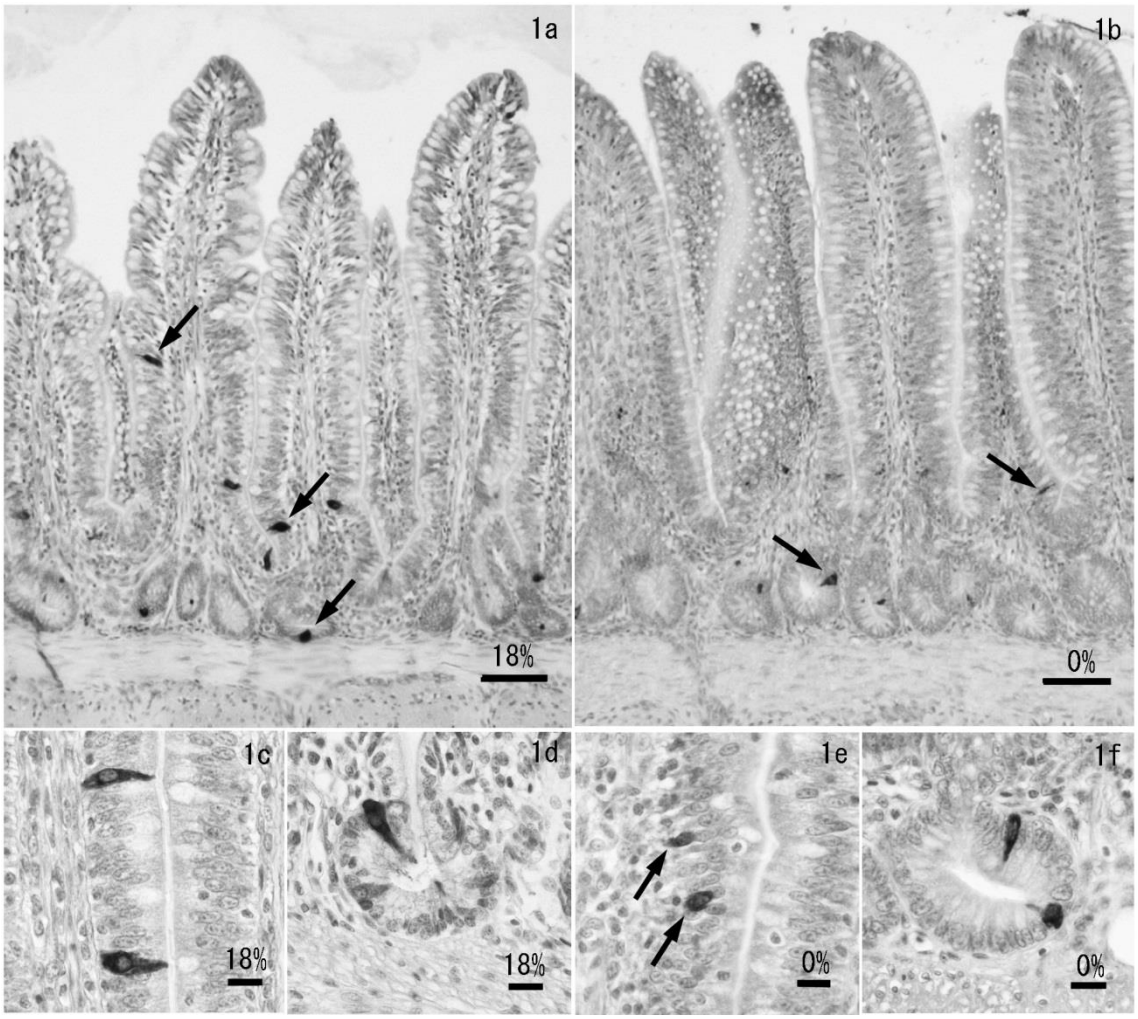
Legends of Figures

Figure 1 GLP-1-imunoreactive cells in the chicken ileum from CP 18% (control: **a, c, d**) and CP 0% (**b, e, f**) groups. **a** Localization of GLP-1-immunoreactive cells (arrows) in the chicken ileum from the CP 18% group. Immunoreactive cells were observed in epithelium of middle and low parts of villi and crypts. Bar: 50 μm. **b** Localization of GLP-1-immunoreactive cells (arrows) in the chicken ileum from the CP 0% group. Immunoreactive cells were mainly observed in crypts. Villous tips tended to be dull compared with those of CP 18% group. Bar: 50 μm. **c, d** High magnification views of GLP-1-immunoreactive cells in villous epithelium (**c**) and crypt (**d**) of ileum from the CP 18% group. They were in contact with intestinal lumen by their cytoplasmic processes. Bar: 10 μm. **e, f** High magnification views of GLP-1-immunoreactive cells in the villous epithelium (**e**) and crypt (**f**) of ileum from CP 0% group. GLP-1-immunoreactive cells (arrows) with a round or oval shape were frequently observed in the villous epithelium of 0% CP level group. Bar: 10 μm.

Figure 2 Frequencies of occurrence of GLP-1-immunoreactive cells in the chicken ileum of four groups (CP 18%, 9%, 4.5% and 0%). Values represent mean number of GLP-1-immunoreactive cells per 1 mm^2 mucosa. There are significant differences between different alphabets ($p<0.05$, n=5, error bars: SD).

Figure 3 Regression line of the frequency of occurrence of GLP-1-immunoreactive cells (Y) on the daily protein intake (X). The equation of this line is $Y = 25.92 + 1.87X$, $p<0.0001$.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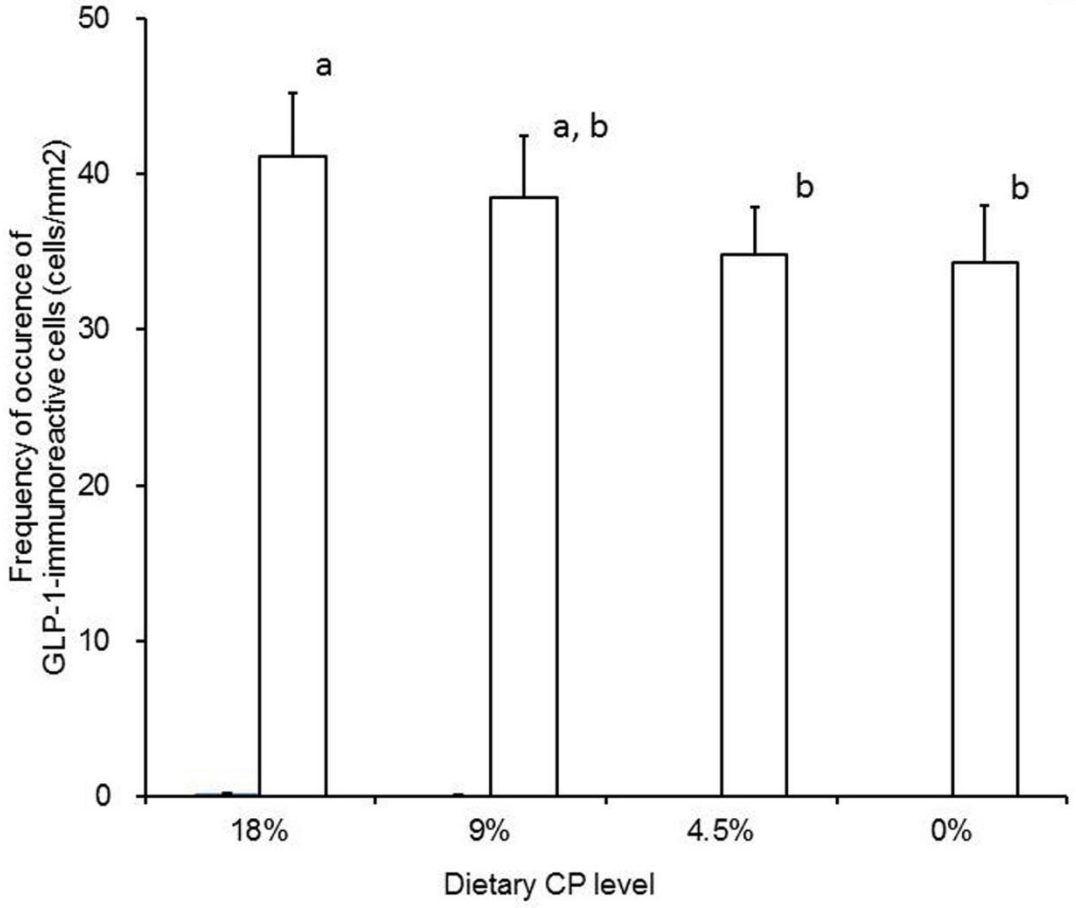
50
40
30
20
10
0
Frequency of occurence of GLP-1-immunoreactive cells (cells/mm2)
a
a, b
b
b
18%
9%
4.5%
0%
Dietary CP level

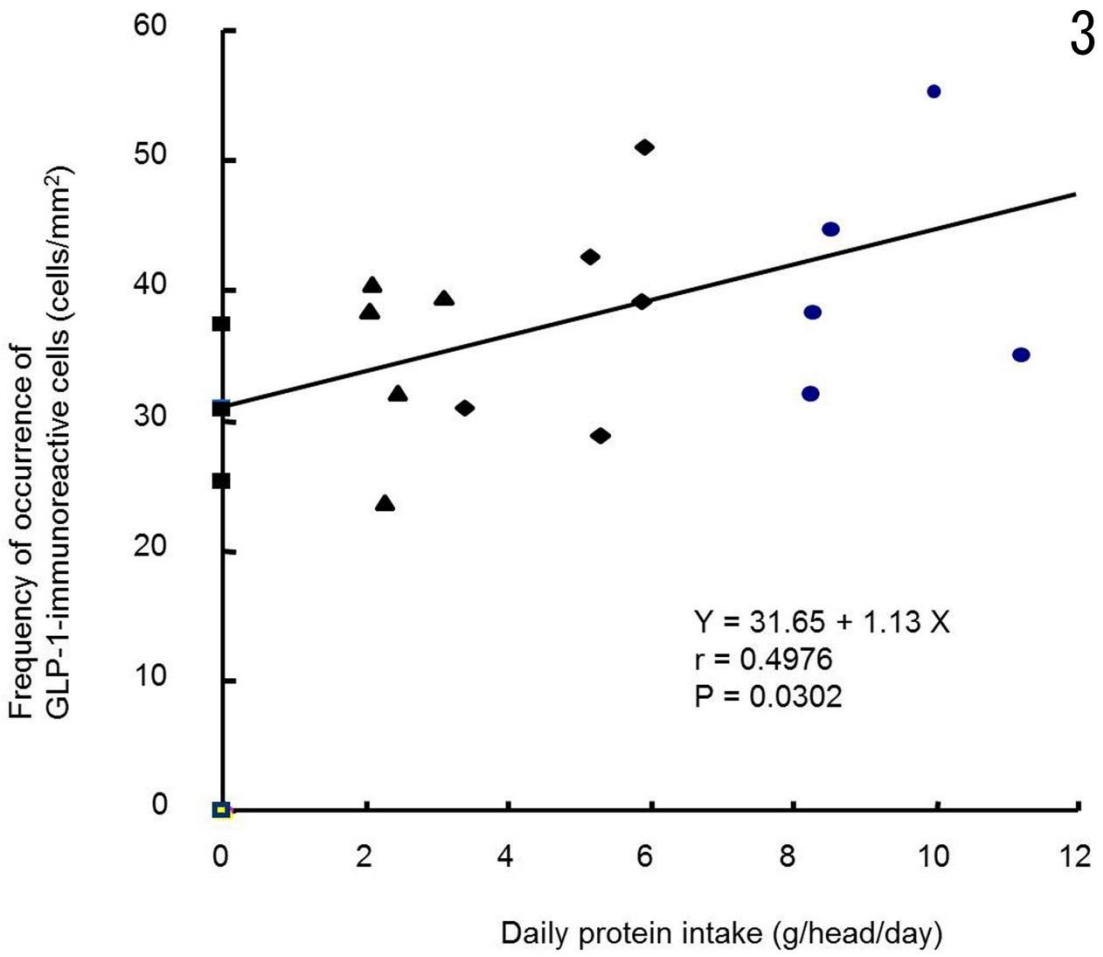
3
Frequency of occurrence of
GLP-1-immunoreactive cells (cells/mm2)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Y = 31.65 + 1.13 X
r = 0.4976
P = 0.0302
Daily protein intake (g/head/day)

Table 1 Compositions of experimental diets used in this study (g). They were composed at the same energy density (metabolizable energy = 2,850 kcal/kg) with the same protein source (ISP: isolated soybean protein).

Composition	CP 18%	CP 9%	CP 4.5%	CP 0%
ISP (CP84%)	214.0	107.0	53.5	0.0
L-Methionine	1.3	0.7	0.4	0.0
L-Cystine	2.2	1.1	0.6	0.0
Cornstarch	493.1	599.1	652.1	705.2
Cellulose	196.4	199.1	200.4	201.8
Corn oil	30.0	30.0	30.0	30.0
Mineral mixture	58.5	58.5	58.5	58.5
Vitamin mixture	2.0	2.0	2.0	2.0
Choline chloride	1.5	1.5	1.5	1.5
Inositol	1.0	1.0	1.0	1.0

Table 2 Body weight gain (g), daily feed intake (g/head/day), daily protein intake (g/head/day) and villous height (μm) of chickens from 4 groups (CP 18%, 9%, 4.5% and 0%). Values are group means±SD. There are significant differences in body weight gain and daily protein intake between different alphabets ($n=5$, $p<0.01$). There are no significant differences in daily feed intake and villous height.

Dietary CP level	18%	9%	4.5%	0%
Body weight gain (g)	96.4 ± 16.1^{a}	78.4 ± 34.7^{a}	28.8 ± 17.6^{b}	-41.4 ± 11.1^{c}
Daily feed intake (g/head/day)	51.4±7.3	57.0±11.5	53.2±9.6	46.9±5.5
Daily protein intake (g/head/day)	9.3 ± 1.1^{a}	5.1 ± 0.9^{b}	2.4 ± 0.4^{c}	0 ± 0^{d}
Villous height (μm)	396.6±72.5	399.3±68.9	379.1±73.8	384.5±66.1