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EFFECT OF *IN OVO* ADMINISTRATION OF BUTYRIC ACID INTO BROILER BREEDER EGGS ON CHICKEN SMALL INTESTINE PH AND MORPHOLOGY

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ABSTRACT

This experiment was conducted to evaluate effects of *in ovo* butyric acid (BA) administration into broiler eggs on chicken small intestine pH and morphology. 480 fertile eggs were obtained from Ross broiler breeder (45 wk) and divided into 3 treatments with 4 replicate and 160 eggs per treatment. On the 18th day of incubation, 1 ml of *in ovo* solution was injected into amniotic fluid. Treatments were including 0.3 % BA solution, 0.9 % NaCl solution and control group. For pH and intestinal morphometric examination, 4 chicks per replicate were euthanized. The results showed that effect of BA injection on jejunum ($p < 0.01$) and ileum pH ($p < 0.05$) on hatch day was significant. Jejunum villi height increased ($p < 0.05$) on the 7th day compared with the control group. The highest ileum villi was observed following the BA injection ($p < 0.01$). It can be concluded that BA injection affects small intestine morphology and increases body weight of chicks.

Key words: *in ovo* injection, butyric acid, pH, small intestine morphology

INTRODUCTION

Butyric acid (CH3CH2CH2COOH; BA) is a medium-chain fatty acid that is absorbed from first section of gastrointestinal.

Pryde *et al*. (2002) reported that BA can be used as an energy source for growth and development of intestine epithelial cells in human. There is not adequate production of BA in the intestine of chick and it is related to the incomplete establishment of gastrointestinal (GI) microbes in the early post-hatch (Hosseini Mansoub *et al.*, 2011). Also the level of short-chain fatty acids in chicken caecum and intestine is very low at early period of life. Dietary supplementation of BA had positive effects on the regulation of intestinal bacterial populations (Bolton and Dewar, 1965) and improved broiler performance and carcass parameters (Leeson *et al*., 2005). It has been shown that BA injection had a significant differences in chick length on hatch day ($P < 0.05$); body weight was increased significantly at the 10th day post hatch by injection of the 0.3 % BA glyceride solution ($P < 0.05$) (Salahi *et al*., 2011). Application of BA (3 g.kg^{-1}) increased chicken small intestine length compared with the control group (Mahdavi and Torki, 2009). BA consumption in chickens had stimulating effects on the intestinal villi (Hosseini Mansoub *et al*., 2011). Addition of 0.2 % mixed triglyceride of BA (mono, two and triglycerides) decreased villi length, increased microvilli length and crypt depth of jejunum (Antongiovanni *et al*., 2007). Application of organic acid in broiler diets decreased intestinal pH. Intestinal pH reduction had beneficial effect on the decreasing of the harmful intestinal bacteria hereby decreasing bacterial fermentation and improving protein and energy digestibility (Adil *et al*., 2011). When pH of digesta was reduced, the pancreatic secretion was increased, and had trophic effects on the mucosa of gastro-intestinal tract. Short chain fatty acids can accelerate gut epithelial cell proliferation, thereby increasing intestinal tissue weight,

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which will result in changes of mucosal morphology and the gastro-intestinal cell proliferation. Based on beneficial effects of BA on broilers' performance we thought that *in ovo* administration of BA could improve the nutritional status during last period of embryonic development and first week after hatch. Therefore, the objective of the study was to determine the effects of dietary administration of BA (HYDRO C4-30) (SILOSPA, Italy) on intestine morphology and pH during last days of incubation and early phase of chicken growth.

MATERIAL AND METHODS

1. Experimental groups

480 fertile eggs were obtained from a 45 wk breeder flock (Ross 308) and then allocated to three treatment groups. These groups included as follows: I (control, non-injected), II (*in ovo* injection of Nacl 0.9 % solution, positive control) and III (*in ovo* injection of 0.3 % BA glyceride solution in 0.9 % saline). Experiments were performed in 4 replicate (40 eggs per replicate) so that totally 160 eggs (66.5 ± 0.5 g) per treatment were used.

2. Butyric acid solution

BA solution used in the experiment was Monobutyrin-Hydro C4-30 (HYDRO C4-30) liquid, water soluble product. The Hydro C4 composition assigned by manufacturer was 47–53 % BA glycerides, 42–46 % free glycerol, 28–32 % BA and 0.5–1 % moisture. pH and osmolarity were 6–7 and 0.017–0.028 mol L^{-1} (0.2–0.33 % solution), respectively and the recommended Hydro C4 in broiler drinking water were 0.2 % to 0.33 % from 0 to 21 days of age (Uni and Ferket, 2003).

3. Incubation and hatching

After 6 hours of injection, the eggs were transferred to hatcher and were set in hatching trays horizontally. Trays were covered by mesh wire to avoid mixing of chicks between adjacent compartments. The hatcher (*Petersime* model 192, analog) temperature and relative humidity were 37.5 °C and 50 %, respectively (Salahi *et al.*, 2011). Chicks were taken-off from hatcher at 508 hours of incubation when 5 % of chicks were still damp at the back of the neck (Kawalilak *et al.*, 2010).

4. *In ovo* injection procedure

Prior to injection, all eggs were candled for examination of embryo position, amniotic fluid and embryo movement, also a Coumassie blue dye solution was injected into the 480 eggs to ensure that the solution is administrated into amniotic fluid. After examination the eggs were *in ovo* injected using 22 needle-gauge at 453 h (18.8 d) of incubation. Solution volume for each injection was 1 ml and needle depth was 2.54 cm (1 inch) (Zhai *et al.*, 2008). After injection, the site of injection was sanitized with ethanol 70 % (Ohta *et al.*, 1999) and sealed with a liquid adhesive.

5. Birds housing

Hatched chicks were raised on floor pens until 10 days. Birds of each pen had *ad libitum* access to similar diet and water. Chicks were raised under similar environmental conditions according to broiler breeder recommendation (Aviagen, 2009) and allocated to pens at 10 m^2 stocking density. Light intensity was maintained 30–40 Lux and 23L: 1D h photo schedule was applied throughout the experiment. No vaccine or drug antibiotics were used during 10 days rearing period.

6. Collection of samples

Upon hatch, 4 chicks were randomly selected and euthanized by CO_2 asphyxiation (Salahi *et al.*, 2011). In this study all chicks were sampled on hatch day (DOH), the 3rd day (D3) and the 7th day post-hatch. The gut samples were taken from the following 3 sites: duodenum (from gizzard until the end of duodenal loop), jejunum (from the end of duodenal loop to Meckel's diverticulum) and ileum (from Meckel's diverticulum to ileo-ceacal junction) (Aviagen, 2009).

7. Processing of Samples and Intestinal Morphology Examination

After opening the abdominal area, the central segment of each sample was extracted and flushed with 0.85 % normal saline (sterile water) to remove the intestinal content (Wang and Peng, 2008); tissue samples (approximately 3 and 2 cm of jejunum and ileum) were obtained from the midpoints of these segments. Furthermore, tissue samples were separated and post fixed in 10 % neutral buffered formalin for histological evaluation (Buchanan *et al.*, 2010). Samples were fixed for 72 h (Kawalilak *et al.*, 2010) and stored in a 70 % alcohol solution until further processing. Each sample was dehydrated, cleared and embedded into paraffin (Wang and Peng, 2008); one 5 μm section of each sample was placed on a glass slide and stained with hematoxylin-eosin and periodic acid-Schiff (PAS) stain. These sections were deparaffinized in xylene and rehydrated in a graded alcohol series (Wang and Peng, 2008). Eight samples obtained for each intestinal tissue and 2 cross-sections were prepared for evaluation. Examination and assays were done under a light microscope (Micros MCX300), photographs were taken of the transverse sections and analyzed using *Ivemp* Image Tool software. Measurement by the software was done on 7 villi per sampled chick (Aptekmann *et al.*, 2001). First, we looked for the highest villi (Kawalilak *et al.*, 2010) and then, selected neighbor of this villi from left side. Pictures of small intestine

villi were acquired under a light microscope with 10× magnification. Villus height was measured from the tip of the villi to the crypt area mouth (Buchanan *et al.*, 2010; Smulikowska *et al.*, 2009) villus-crypt junction (Wang and Peng, 2008) or the top of the *lamina propria* (Aptekmann *et al.*, 2001). Another morphometric variables were included; villus width was measured at half height in the middle of its length) Buchanan *et al.*, 2010; Smulikowska *et al.*, 2009). The crypt depth was defined as from the base upwards to the region of transition between the crypt and villus (from crypt mouth to base) (Aptekmann *et al.*, 2001; Smulikowska *et al.*, 2009). Villus apical width at the villus tip, basally to the crypt-villus junction, was measured, then villus surface area was estimated by the following mathematical formula; [((villus tip + villus base/ 2)× villus height [((Incharoen and Yamauchi, 2009). Finally, villus height: crypt depth ratio and crypt: villus height ratio were calculated.

8. pH Measurement

pH was measured in jejunum and ileum segments of the gastrointestinal tract (GIT) of broiler chickens at different ages including the 3rd and 7th day of the post-hatch .In this study we measured pH using Ex-situ protocol (inserting the pH probe). Then intestinal contents collected and transferred into 3 ml of distilled water (Chaveerach *et al.*, 2004). pH measurement was done by PH/°C meter (A∂WA AD 110, Romania) and sensor was placed for 20 second into the solution.

9. Statistical Analysis

The experiment had a completely randomized design with 3 treatments; each of them applied to the same number of units. The data were subjected to statistical analysis based on 4 replications per treatment. One-way ANOVA was performed to examine differences among groups by using the SAS software (SAS Institute Inc, 2004). The means of variables were compared using Duncan's multiple range test.

RESULTS

Results of this study showed that effect of *in ovo* injection of different treatments on the pH of jejunum was significant on hatch day (p <0.01) so the lowest pH was observed by injection of 0.3 % BA .Jejunum pH at the 3rd and 7th days after the hatch was not affected by different treatments. Also ileum pH at the hatch day was affected by different treatments (p <0.05) and the lowest pH was observed in ileum by injection of 0.3 % BA (Table 1). Jejunum villi height was affected at the hatch day (p <0.001) and the maximum jejunum villi height was observed on the control of the group. Also jejunum villi height on the 7th day of the post-hatch was affected by different treatments (p <0.05), so the maximum height was observed in the Nacl and BA injection groups, respectively. Jejunum villi width on the 3rd and 7th days of the post-hatch on BA injection treatment was higher than in other groups, although this effect was not statistically significant. Jejunum crypt depth at hatch day and the 3rd day after the hatch on BA injection group were larger than in other groups, although this effect was not significant (Table 2) too. The ileum villi height on the hatch day was the impact of different treatments (p <0.01) and the maximum height was observed in the BA treatment. Ileum villi width on the 7th day of the post-hatch in BA injection group was more than in other groups, also this difference was not significant too. However, ileum crypt depth on the 3rd and 7th days of the post-hatch in the BA treatment were larger than in other groups, differences were observed between treatments, but this effect was

Table 1: Effects of BA injection on small intestine pH

Treatment	Control	NaCl 0.9 %	BA 0.3 %	SEM	*p*-value
			Jejunum pH		
Hatch day	6.9[a]	6.8[b]	6.7[c]	0.04	0.01
3 Day	5.3	4.9	4.7	0.26	0.28
7 Day	5.67	5.58	5.8	0.21	0.59
			Ileum pH		
Hatch day	7.06[ab]	7.2[a]	6.8[b]	0.09	0.05
3 Day	5.91	5.96	5.93	0.13	0.67
7 Day	5.85	5.79	6.02	0. 15	0.68

[a–c] within main effects, values followed by different letters within columns are significantly different (P <0.05*); SEM – Standard Error of Mean

Table 2: Effects of BA injection on jejunum morphology

Treatment	Control	NaCl 0.9 %	BA 0.3 %	SEM	*p*-value
Jejunum Villus height (µm)					
Hatch day	473[a]	460[b]	458[c]	8	0.001
3 Day	963	906	888	76	0.73
7 Day	1 231[b]	1617[a]	1426[ab]	83	0. 02
Jejunum villus width (µm)					
Hatch day	130	114	96	12.1	0.18
3 Day	167	153	168	20.5	0.72
7 Day	188	211	237	19	0.46
Jejunum Crypt depth (µm)					
Hatch day	147	161	158	16.4	0.81
3 Day	228	248	257	17.1	0.64
7 Day	383	324	344	26.4	0.13

[a–c] within main effects, values followed by different letters within columns are significantly different ($P < 0.05$*); d – day; J – Jejunum

Table 3: Effects of BA injection on ileum morphology

Treatment	Control	NaCl 0.9 %	BA 0.3 %	SEM	*p*-value
Ileum villus height (µm)					
Hatch day	378[c]	409[b]	414[a]	6	0. 01
3 Day	597	600	602	33	0. 84
7 Day	955	937	1 064	76	0. 53
Ileum villus width (µm)					
Hatch day	109	100	101	7.6	0.34
3 Day	159	144	156	13.7	0.38
7 Day	214	200	238	24	0.55
Ileum Crypt depth (µm)					
Hatch day	153	171	176	12.1	0.65
3 Day	222	210	233	21. 8	0.74
7 Day	267	291	297	22.6	0.46

[a–c] within main effects, values followed by different letters within columns are significantly different ($P < 0.05$*)

not statistically significant (Table 3). Jejunum villi surface area was not affected by different treatments. Ileum villi area on the hatch day and the 7th day of the post-hatch on BA injection treatment were larger than in other groups, but there was no significant difference between treatments. Crypt to villi ratio in the jejunum on the 7th day of the post-hatch was influenced by different treatments ($p < 0.05$) and in control, and BA groups were more than Nacl group, but this was not significant. Crypt to villi ratio in ileum on the hatch, 3rd and 7th days of the post hatch were not affected by different treatments (Table 4).

Table 4. Effects of BA injection on Villus Area and Crypth to villi ratio

Treatment	Control	NaCl 0.9 %	BA 0.3 %	SEM	*p*-value
Jejunum Villus Surface Area (μm²)					
Hatch day	25 833	23 953	22 561	3 800	0. 84
3 Day	51 223	50 832	49 787	8 100	0. 98
7 Day	82 369	112 162	102 796	12 600	0. 74
Ileum Villus Surface Area (μm²)					
Hatch day	14 177	14 318	15 934	943	0.44
3 Day	37 291	36 781	34 092	636	0.74
7 Day	70 640	72 230	79 520	515	0.62
Jejunum Crypt to villi ratio					
Hatch day	0.29	0.33	0.42	0.05	0.21
3 Day	0.24	0.27	0.29	0.02	0.35
7 Day	0.31[a]	0.21[b]	0.24[ab]	0.02	0.03
Ileum Crypt to villi ratio					
Hatch day	0.41	0.43	0.42	0.06	0.65
3 Day	0.38	0.33	0.38	0.04	0.61
7 Day	0.28	0.32	0.28	0.03	0.46

[a–b] within main effects, values followed by different letters within columns are significantly different (P <0.05*)

DISCUSSION

1. pH of intestine

Our findings showed that injection of BA affected the pH of jejunum and ileum on the hatch day with a decrease in the pH of the jejunum. Many studies showed that organic acids have effects on the pH of gastro-intestinal tract (GIT). These studies reported the inclusion of organic acids in the diets of broiler chickens diminished pH of the GIT (Adil *et al.*, 2008) and crop, but no effect on the caeca (Bolton and Dewar, 1965). pH variability between birds was high, but standard deviation between segments was low (Angel *et al.*, 2010). These observations were consistent with Paul *et al.* (2007). This is resulted from strong buffering action of the GI tract in poultry. In the present study, we found no significant effects of BA on pH at the 3rd and 7th days. Therefore, age may not have this significant effect on pH of the ileum and jejunum at the 3rd and 7th day in broilers. The finding is in agreement with Angel *et al.* (2010), who suggested that age had no effects on pH at days 5, 18, 46. They reported the flowing pH for different segments of the GIT: duodenum (5.99, 5.87), jejunum (6.07, 5.97), ileum pH (7.12, 7.15), crop (5.32, 5.85), gizzard (2.37, 2.64, 2.04) and proventriculus (2.14, 2.50, 2.36). Gastric reducing pH increases activity of pepsin, and pepsin proteolysis may cause an increase in peptides. This results in the release of hormones including gastrin and cholecystokinin, (CCK) and these hormones can regulate protein digestion and absorption physiological regulators of feeding, feeding behavior and emotional responses in the central nervous system by affecting gallbladder contractions, exocrine pancreatic secretions and gastric acid secretions in the GIT (Noble and Roques, 1999). Also CCK modulated gastro-duodenal and intestinal motility in chickens (Martinez *et al.*, 1995). Another advantage of reducing pH was direct antimicrobial effects on intestinal bacteria by two methods: penetrating the bacteria cell wall and disrupting the normal physiology of certain types of bacteria when it was used in non-dissociated (non-ionized, more lipophilic) form and reducing bacterial competition with the host for available nutrients and diminution in the level of toxic bacterial metabolites as a result of lessened bacterial fermentation which results in the improvement of protein and energy digestibility, thereby improving the weight gain and performance of broiler (Adil *et al.*, 2011). These effects will ultimately result in a better performance.

2. Jejunum Morphology

Jejunum villi height was affected at the hatch (P <0.001) and different treatments affected (P <0.05) the

height at the 7th day of the post-hatch. This finding is an agreement with other experimental studies. Addition of 1 % of organic acid mixture significantly increased villus height, compared with 0 and 0.5 % addition of organic acid mixture at the 21st day of age (P <0.05) (Saki *et al.,* 2011). Dietary supplementation of organic acids significantly (P <0.05) increased the villus height in the duodenum and jejunum and the highest duodenal and jejunal villus heights were observed in bird fed diets supplemented with 3 % BA and 3 % fumaric acid (Smulikowska *et al.*, 2010). Jejunal villus height was higher (P <0.05) in formate + propionate supplemented group. This result revealed that combination of supplemental acidifier improved or maintained similar villus height when compared with antibiotic supplementation. The increase in villus height of different segments of the small intestine may be attributed to the fact that the intestinal epithelium acts as a natural barrier against pathogenic bacteria and toxic substances that are present in the intestinal lumen (Paul *et al.*, 2007). The villus height in the BMD (bacitracin methylene disalicylate) group of birds was higher than the rest of the treatment group in the jejunum where maximum digestion and absorption takes place as there is a large luminal site, and more mature enterocytes are present. The higher villus height coupled with a higher Lactobacillus count caused a better body weight and FCR in the organic acid blend dietary groups. In the present study, jejunum villi width between the 3rd and 7th days of the post-hatch in butyric acid injection treatment was higher than in other groups, although this effect was not statistically significant. These effects, especially the observed increase in duodenal villous length, are potentiated when the probiotics are used and antibiotics induce enlargement of villous length and width (Markovic *et al.*, 2007). We also observed that jejunum crypt depth on the day of hatch and the 3rd day after the hatch in BA injection group was higher than in other groups, although this effect was not significant. Crypt depth was increased by addition of 0.5 and 1 % of organic acid on the 21st and 42nd days of age (P < 0.05) (Saki *et al.*, 2011). Effects of organic acid on the crypt depth in the duodenum and jejunum did not differ among different treatment groups (Smulikowska *et al.*, 2010). The crypt depth in the duodenum and jejunum was not affected among different treatment groups (Adil *et al.*, 2010). The crypt can be regarded as the villus factory and a large crypt indicates rapid tissue turnover and a high demand for new tissue (Choct, 2009). *In ovo* injection of BA proved that jejunum villi surface area was not affected by different treatments. Crypt to villi ratio in the jejunum on the 7th day of the post-hatch was influenced by different treatments (p <0.05) and in control and butyric acid injection group more than Nacl injected group, but this was not significant. This finding is in contrast with Saki *et al*. (2011) who found that villus surface was significantly increased by addition of 1 % of organic acid mixture compared with 0 % level on the 21st day of age. These histological changes probably had increased the intestinal surface area, facilitating the nutrient absorption to a greater extent and boosted the growth promoting effect of the organic acid supplementation (Smulikowska *et al.*, 2010). It is well known that the presence of increased villus height suggests an increased surface area capable of greater absorption of nutrients (Caspary, 1992).

3. Ileum Morphology

The ileum villi height on the hatch day was affected by different treatments (P <0.01) and the maximum height was observed in the treatment with BA injection. Ileum villi width on the 7th day of the post-hatch in butyric acid injection group was higher than in other groups and this difference was not significant. In other studies, dietary supplementation of organic acids increased the villus height in the ileum but the values were significant (P <0.05) (Smulikowska *et al.*, 2010). Addition of organic acids increased villus height in the small intestine but the differences were not significant in case of the ileum (Adil *et al.*, 2010). The highest ileal villus heights were recorded in the bird fed diets supplemented with 2 % fumaric acid (Smulikowska *et al.*, 2010). More effects on ileum villi height were observed in organic acid levels especially at 1 %, which resulted in an increase in lactic acid bacteria and decrease in Enterobacteriaceae counts in the ileum of broiler chicken (P <0.05) (Saki *et al.*, 2011). We found that ileum crypt depth on the 3rd and 7th days of the post-hatch in the BA treatment was higher than in other groups but differences between treatments were not significant. Using of the organic acid did not result in any effect on the crypt depth in the ileum among different treatment groups (Smulikowska *et al*., 2010). The crypt depth in the ileum was not affected among different treatment groups (Adil *et al*., 2010). In the current study, ileum villi area on the hatch day and the 7th day of the post-hatch on butyric acid injection treatment was higher than in other groups, but there was no significant difference. Dunham *et al.* (1993) presented similar conclusions in domestic fowl fed a diet supplemented with L. Reuteri (probiotics) which had longer villi and lower crypts in ileum compared to the controls. The ileal crypt depth was decreased by synbiotic supplementation (117 ± 2 μm) compared with control (128 ± 2 μm); the addition of synbiotic increased (P <0.001) the villus height/ crypt depth ratio and villus height in ileum compared with control (Awad *et al.*, 2008). Our findings showed that crypt to villi ratio in ileum on the hatch day, 3rd and 7th days of the post-hatch was not affected by different treatments. It can be concluded that BA injection during embryonic period via egg can increase the absorption

area by improving the small intestine morphology, so that it may have effect on the increase in body weight. Also pH of jejunum and ileum at hatch day was affected by *in ovo* injection and the lowest pH was observed in ileum and Jejunum by injection of 0.3 % BA.

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