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# Dietary Betaine Affect Duodenal Histology of Broilers Challenged with a Mixed Coccidial Infection

[1]H. Hamidi, [1]J. Pourreza and [2]H. Rahimi
[1]Department of Animal Science, Faculty of Agriculture, Isfahan University of Technology, Isfahan, Iran
[2]Infection Diseases Unit, Department of Pediatric, Isfahan University of Medical Science, Isfahan, Iran

**Abstract:** The purpose of this research was to investigate effect of dietary betaine on intestinal morphology after an experimental coccidiosis. Hence a total of 189 male and female broiler chicks were randomly assigned to 9 floor cages. Chicks were fed a basal diet supplemented with 0, 0.6 or 1.2 g $kg^{-1}$ betaine. All birds were inoculated orally with *Eimeria* oocysts on day 28. Duodenal morphology parameters and lesions were scored by microscopic observation on intestine samples which were taken at day 42 of age. Adding 1.2 g $kg^{-1}$ betaine to diet diminished intestinal lesions ($p<0.05$). Dietary supplementation with 0.6 or 1.2 g $kg^{-1}$ betaine significantly ($p<0.01$) increased intraepithelial lymphocytes as well. Level of additive betaine had no effect on the ratio of villus height/crypt depth or villus surface area. Lamina propria of duodenum became thicker in the intestine of chickens which received more supplemental betaine via their diet. In conclusion, since the number of intraepithelial lymphocytes and thickness of lamina propria represent the condition of gut immune response, it seems that dietary betaine may immunomodulate the gastrointestinal tract of broilers. In addition, betaine effect on villus morphology measured later in life differed from what had been measured already earlier in life of the chicks.

**Key words:** Broiler, betaine, intraepithelial lymphocyte, villus morphology

## INTRODUCTION

The intestinal villus and crypt morphology in chickens have been associated with intestine function and chicken growth. Research on villus morphology (Tarachai and Yamauchi, 2000) illustrated that villus morphology is governed by enteral nutrient absorption.

Coccidiosis, caused by the microparasites of the genus *Eimeria*, is the prevalent pathogens of chickens and an economically important intestinal infection of poultry. The lesions induced by pathogen are visible both macroscopically and microscopically. Furthermore, coccidian infection associated with metabolic and structural changes in the intestinal mucosa of the host animal (Waldenstedt *et al*., 1999a). Dietary betaine has shown that could stabilize the intestinal epithelial structure in healthy and coccidia-infected birds (Kettunen *et al*., 2001). It is possible that the action of betaine is related to its influence on antibody production or phagocytes because these cells are important in protection against coccidia (Yun *et al*., 2000). On the other hand, the influence of betaine as an osmolyte can assist to improve work and stability of infected intestinal mucous (Allen *et al*., 1998; Allen and Fetterer, 2002).

Klasing *et al.* (2002) reported that the number of intraepithelial lymphocyte and thickness of lamina propria were increased by supplemental betine. Kettunen *et al.* (2001) also observed that the crypt/villus ratio was decreased by the dietary betaine supplementation in healthy and coccidia-infected chicks. Moreover, have been reported inconsistent results of betaine effect on intestinal lesions.

It is probable that some of the morphological parameters of intestine be affected by age, so that rapid growth rate and morphological changes of gastrointestinal track during several early weeks of age (Nitsan *et al*., 1991) interfere with morphological measurements. Even it has been demonstrated (Iji *et al*., 2001) that crypt depth and villus height had interaction with age of chickens especially in early weeks. Moreover, practically infection of broilers with coccidiosis predominantly occurs after the 4th (Biffa *et al*., 2003) or even 6th (Malcolm, 1978) week of age, when the infection has maximum economical and physiological harms, because of short time to redress. In addition Malcolm (1978) described that the appearance of new generation of *Eimeria* oocystes is relatively late in the life cycle, usually a week after ingestion of sporulated oocystes.

**Corresponding Author:** H. Hamidi, Department of Animal Science, Faculty of Agriculture, Isfahan University of Technology, P.O. Box 84154, Isfahan, Iran

However, almost all of previous studies have examined morphological effect of betaine in coccidiosis condition during 14 to 21 day period. Thus in this research morphological effect of betaine at day 42 was examined on the intestine of chickens which challenged by coccidian-infection at day 28 of age to compare intraepithelial lymphocytes and thickness of lamina propria as indicators of gut immune response as well as intestinal lesions and villus dimension with earlier studies.

## MATERIALS AND METHODS

**Diet, birds and experimental design:** The presented study was performed in the experimental farm of Isfahan University of Technology, Isfahan, Iran. A total of 189 male and female seven day-old broiler chicks (Ross 308) were randomly assigned to 9 floor cages. Chicks were fed a basal diet supplemented with 0, 0.6 or 1.2 g kg$^{-1}$ betaine. A complete random design was used with 3 replicates during the 7 to 42 day period. The diets were formulated to meet National Research Council (1994) nutrient requirements (Table 1). The chickens were fed starter diet ad libitum until day 21 and grower diet offered from 21 to day 42. Diets contained no coccidiostats.

**Experimental infection and tissue sampling:** At day 28 of age, all birds were inoculated orally with a mixed culture of *Eimeria tenella* and *Eimeria acervulina* to simulate a coccidiosis challenge by $8.5\times10^3$ and $7.5\times10^3$ sporulated oocysts per chicken respectively. On day 42 the duodenum sections were taken from ~2 cm to the duodenal loop at the apex of the pancreas. Segments of ~1.5 cm in length were flushed with saline and fixed in 100 g L$^{-1}$ buffered formalin (pH 7.0). The fixed intestinal samples embedded in paraffin wax then were sectioned (5 μm) and stained with hematoxylin-eosin finally were examined by light microscope for the following: lesions score, number of intraepithelial lymphocytes, villus height/crypt depth (V/C) ratio (villus height; from the top of the villus to the top of the lamina propria and crypt depth; from the base upward to the region of transition between the crypt and villus), villus surface area and lamina propria thickness (space between the base of the villus and the top of the muscularis mucosa) (Aptekmann *et al*., 2001). The tissue morphology and lesions was graded (Zentek *et al*., 2002) by one person without the knowledge of the origin of the sample. Comparative scores were from 1 to 4 including mediate numbers (1, 1.5, 2, 2.5,..., 4). Data were collected on 10 different villi per chicken on sections of both two chickens per pen.

Table 1: Ingredients and calculated nutrient content of the basal starter and grower diets

| | Day period | |
|---|---|---|
| Ingredients (%) | 7 to 21 | 21 to 42 |
| Corn | 55.56 | 64.23 |
| Soybean meal (44%) | 34.04 | 29.39 |
| Fish meal | 3.50 | 1.00 |
| Fatty acid | 3.50 | 2.00 |
| Dicalcium phosphate | 1.01 | 1.12 |
| Oystershell | 1.39 | 1.39 |
| D-L methionine | 0.14 | 0.03 |
| Vitamin premix[A] | 0.25 | 0.25 |
| Mineral premix[B] | 0.25 | 0.25 |
| NaCl | 0.24 | 0.22 |
| Variable[C] | 0.12 | 0.12 |
| **Energy and calculated nutrient content** | | |
| Energy (kcal ME kg$^{-1}$) | 2991.00 | 2990.00 |
| Crude protein (%) | 21.52 | 18.67 |
| Lysine (%) | 1.25 | 0.93 |
| TSAA[D] (%) | 0.87 | 0.67 |
| Calcium (%) | 0.93 | 0.81 |
| Available phosphorus (%) | 0.42 | 0.37 |

A: Vitamin premix provides the following per kilogram: vitamin A 8800 IU; cholecalciferol 3300 IU; vitamin E 16.53 IU; vitamin B 0.023 mg; riboflavin 8 mg; niacin 33 mg; pantothenic acid 35 mg; menadione 1.5 mg; folic acid 0.8 mg; thiamin 3 mg; pyridoxine 2.7 mg; biotin 0.25 mg; ethoxyquin 125 mg; B: Mineral premix provides the following in milligrams per kilogram: Mn, 55; Zn, 50; Fe, 50; Cu, 5; Se, 0.1; I, 1.5, C: Variable amounts of betaine and washed builders sand, D: TSAA: Total sulfur amino acids

**Statistical analysis:** Data were analyzed using the GLM procedures of SAS Institute (SAS, 1999). Comparisons among the means were made using Dancan's multiple range test procedure. The means differences were considered significant at a probability $p<0.05$.

## RESULTS AND DISCUSSION

Statistical analysis of data indicated that: there were significantly ($p<0.05$) less intestinal lesions in chickens which received 1.2 g kg$^{-1}$ betaine, compared to those unsupplemented with betaine (Table 2). Despite no statistically significant difference of lesion scores between 0 and 0.6 g kg$^{-1}$ betaine levels, decreasing intestinal lesions numerically was observable. This result is in agreement with findings by Tiihonen *et al.* (1997) and Hess *et al.* (1998), who reported that morbidity and macroscopic intestinal lesions resulting from infection with *Eimeria* diminished by dietary betaine. Also Virtanen *et al.* (1996) reported a decrease in lesion score of chicks fed betaine in combination with salinomycin, whereas Remus and Virtanen (1996), Zimmermann *et al.* (1996) and Matthews *et al.* (1997) didn't report this effect for betaine added in diet.

The number of intraepithelial lymphocytes in the intestinal section of birds whose diet was supplemented with 0.6 or 1.2 g kg$^{-1}$ betaine was significantly ($p<0.01$) greater than those receiving no betaine (Table 2).

Table 2: Effect of betaine levels on histological and morphological parameters

| Betaine (g kg$^{-1}$) | Lesions score | Intraepithelial lymphocytes | V/C | Villus surface area | Lamina propria thickness |
|---|---|---|---|---|---|
| 0.0 | 3.25$^{a}$ | 1.25$^{b}$ | 3.25$^{a}$ | 2.67$^{a}$ | 1.42$^{c}$ |
| 0.6 | 2.42$^{ab}$ | 2.58$^{a}$ | 3.00$^{a}$ | 2.25$^{a}$ | 2.17$^{b}$ |
| 1.2 | 2.00$^{b}$ | 2.83$^{a}$ | 3.17$^{a}$ | 2.58$^{a}$ | 3.00$^{a}$ |
| SEM | 0.193 | 0.250 | 0.197 | 0.246 | 0.222 |
| Probability | * | ** | NS | NS | *** |

Values are means of comparative scores, V/C: Villus height/Crypt depth, SEM: Standard error of means, $^{a-b}$Values within variables with no common superscripts differ significantly ($p<0.05$), NS: Not Significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$

Klasing *et al*. (2002) reported the same results with adding 0.5 or 1 g kg$^{-1}$ betaine to the diet. They suggested that increased chemotaxis of monocytes and nitrogen oxide release by macrophages may explain the decreased intestinal pathology but increased leukocyte numbers that were observed when betaine was fed during a coccidia infection. Intestinal lymphocytes are present in two anatomic compartments within the intestinal mucosa, the epithelium and lamina propria (Befus *et al*., 1980) which these cells and T cells regulate mucosal immune responses. In addition, intraepithelial lymphocytes have been demonstrated to secrete several cytokines, including interleukins, transforming growth factor and interferon-γ (Mayer *et al*., 1991; Lundqvist *et al*., 1996; Fan *et al*., 1998). Furthermore chicken intestinal intraepithelial lymphocytes contain a subpopulation of Natural Killer (NK) cells that serve an important role in controlling coccidiosis, perhaps by cytolysis of infected cells or as a source of interferon (Lillehoj, 1989). Hence these cells are an important part of intestinal immune responses. According to results of this study, dietary supplementation with betaine leads to the improvement of this immune criterion.

As shown in Table 2 the ratio of V/C was unaffected by betaine level. However, Klasing *et al*. (2002) reported that villi height was decreased by coccidiosis and this was ameliorated by 1.0 g kg$^{-1}$ dietary betaine. Also Kettunen *et al*. (2001) observed that the crypt/villus ratio was decreased by the dietary betaine supplementation in healthy and challenged chicks. The sensitivity of the V/C ratio has been demonstrated even after alterations in the lipid composition of the diet (Sagher *et al*., 1991; Galluser *et al*., 1993), dietary supplemental calcium (Aptekmann *et al*., 2001) and Zn deficiency in the diet (Southon *et al*., 1985). Indeed, in relation to villus height and crypt depth, the important point is that the proliferation in the small intestine increases the crypt proliferative compartment. Instead, the absorptive villus compartment becomes smaller. Consequently, nutrient absorption would be affected.

A more reliable criterion for absorptive area than V/C ratio is villus surface area because this parameter includes villus height, width and amount of folds on villus. Thus in this research, villus surface area was examined to detect more precise effect of betaine (if there be any) on absorptive area. As shown in Table 2 there was no significant difference in duodenal villus surface area between betaine levels. Reduction of villus surface area, in coccidiosis condition, is thought to be responsible for nutrient malabsorption -a typical symptom of coccidiosis (Scott *et al*., 1982; Girdhar *et al*., 2006). One result of nutrient malabsorption is the reduction of feed efficiency. According to the presented results, it appears that the improvement of feed efficiency has been observed in some experiments (Waldenstedt *et al*., 1999a, b) in which coccidia-infected chicks were dietary supplemented with betaine is not due to the effect of betaine on absorptive surface. Probably, stabilizery and osmoregulatory effects of betaine are responsible for it.

Thickness of lamina propria was increased highly significantly by the elevation of dietary supplemental betaine level so that level of 0.6 g kg$^{-1}$ betaine differed significantly from 0, as well as 1.2 from 0.6 (Table 2). This result supports what has been reported by Klasing *et al*. (2002). Thickness of lamina propria could be an indicator of gut health, because it contains dendritic cells that survey the contents of the lumen and protect the chicken from infection by stimulating the adaptive immune response, increasing gut motility and modifying mucin production, defensin secretion and immunoglobulin A (IgA) production. On the other hand, only a layer of mucin and a single cell layer of enterocytes separate the deeper tissues of the body from potential opportunistic pathogens found in the lumen of the gastrointestinal tract (Macpherson and Harris, 2004).

## CONCLUSION

In conclusion, according to beneficial effect of dietary betaine on intraepithelial lymphocytes and thickness of lamina propria observed in this research and the importance of these tow parameters in immunocompetence of birds it seems that dietary betaine may immunomodulate the gastrointestinal tract of broilers. Furthermore, betaine effect on villus morphology measured later in life differed from what had been measured already earlier in life of the chicks. However, betaine didn't expand the absorptive surface of the villus.

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