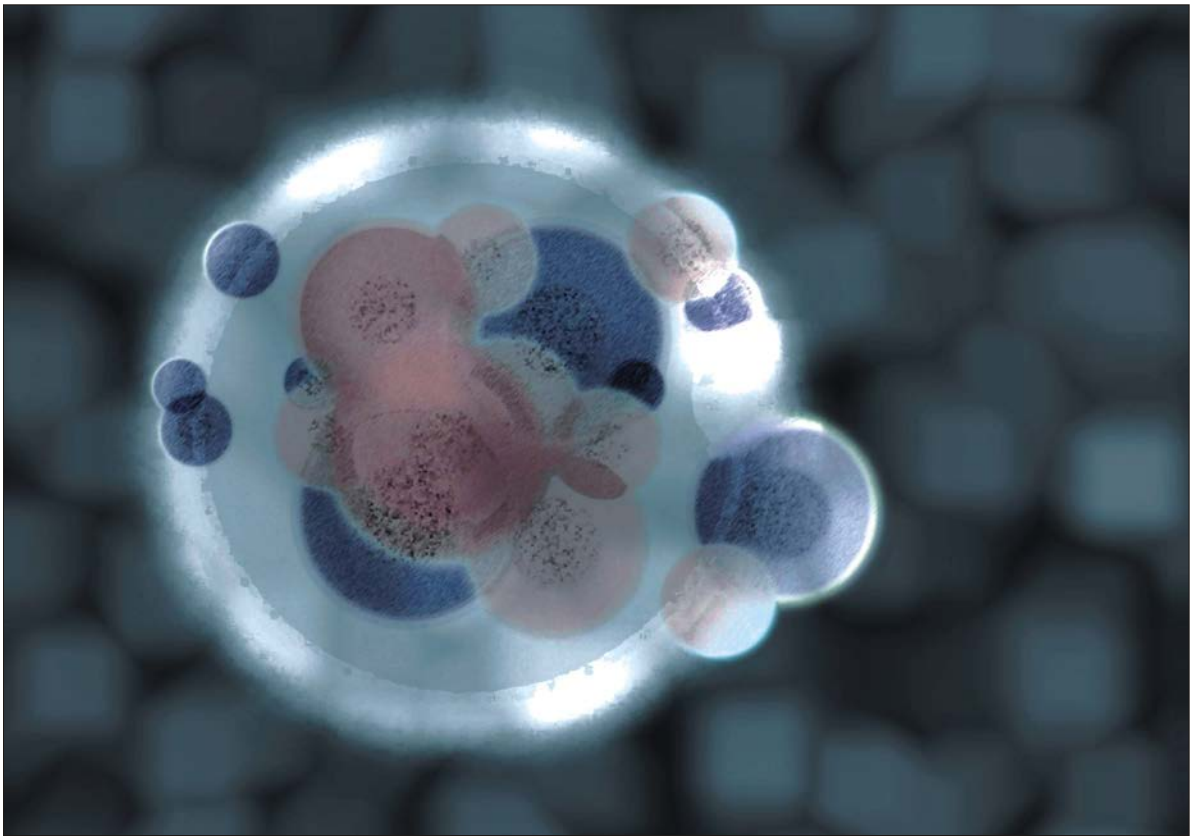

Journal of Biological Sciences

ISSN 1727-3048

Journal of Biological Sciences 9 (7): 760-765, 2009
ISSN 1727-3048

Dietary Zinc-Methionine and Feed Restriction Affect Duodenal Morphology of Broilers Challenged with a Mixed Coccidial Infection

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Abstract: The purposes of this study was to investigate effects of additive zinc-methionine (Zn-Met) in a diet contained practical level of Zn and alternate day feeding as physical Feed Restriction (FR) early in life on intestinal morphology after an experimental coccidiosis. Hence, a total of 378 male and female broiler chicks were fed a basal diet (74 mg Zn kg^{-1}) supplemented with 0, 40 or 60 mg Zn kg^{-1} as Zn-Met which was administrated *ad libitum* (AL) or with feed restriction. Feed restriction chicks consumed food on alternate days from 11 to 18 day of age. All birds inoculated orally with two species of coccidia on day 28. Duodenal morphology and lesions were scored by microscopic observation on duodenal samples which were taken at day 42 of age. Intestinal lesions were diminished ($p<0.01$) by dietary supplementation with 40 or 60 mg kg^{-1} Zn-Met. Also, number of intraepithelial lymphocytes was significantly ($p<0.05$) increased by 60 mg kg^{-1} level. But administration of FR could affect neither lesion score nor intraepithelial lymphocyte. The ratio of villus height/crypt depth (V/C) was larger in broiler chicks which received 60 mg kg^{-1} Zn-Met than unsupplemented group. Birds in FR group had more expanded villus surface ($p<0.01$) and thinner lamina propria ($p<0.001$) than AL group. Increase of intraepithelial lymphocytes as an indicator of intestinal immune response and decrease of duodenum lesions in chickens which dietary supplemented with Zn-Met suggest that preparing more available zinc in broilers diet might be necessary to maximize immunocompetence of broilers. Also, alteration of V/C ratio and villus surface area by additive Zn-Met and FR may cause to improve efficiency of nutrients utilization in coccidiosis condition.

Key words: Zinc-methionine, feed restriction, intraepithelial lymphocyte, villus morphology, coccidiosis

INTRODUCTION

It has been reported that zinc level of chick diet in practical situations is not low enough to induce immune changes (Pimentel *et al*., 1991a). And it has been suggested that the immune responses are less sensitive to Zn intake than growth of chicks (Pimentel *et al*., 1991b). However, some studies have reported that supplementation of diets, which contain sufficient level of Zn -according to NRC (1994) recommendations- with zinc-methionine (Zn-Met) had beneficial effects on some different immune responses including cellular and humoral immunity (Hudson *et al*., 2004; Kidd *et al*., 2000; Virden *et al*., 2002; Hamidi and Pourreza, 2009) nevertheless some other researchers reported in disagreement. Some of this kind studies have been reviewed by Kidd *et al*. (1996), who suggested that: in order to determine zinc or Zn-Met requirements for broiler chick immune responses, different criteria may be necessary. It's likely that much more supplemental Zn could improve some criteria of immunity.

The intestinal villus and crypt morphology in chickens has been associated with intestine function and chicken growth. Researches on villus morphology (Shamoto *et al*., 1999; Shamoto and Yamauchi, 2000) suggested that luminally absorbed nutrients may influence villus morphology. Hence, Tarachai and Yamauchi (2000) illustrated that villus morphology is governed by enteral nutrient absorption. Also, villi height (Yamauchi *et al*., 1995, 1996) and cellular proliferation within the crypt (Yamauchi *et al*., 1996; Shamoto *et al*., 1999) in the duodenum showed a rapid decrease within the first 24 h of feed withdrawal but recovered rapidly after refeeding. On the other hand, Feed Restriction (FR), in experimental infections with *Eimera tenella* (Zulkifli *et al*., 1993) and *E. coli*. (Boa-Amponsem *et al*., 1997) has showed beneficial effects on resistance against these pathogens. As well as on the elevation of immunoglobulin A production (Hamidi and Pourreza, 2009) and antibody response to sheep red blood cell (Khajavi *et al*., 2003). Thus, we were interested in investigating whether alternate day feeding as physical

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feed restriction early in life would affect intestinal morphology and gut health indicators (intestinal lesion score, intraepithelial lymphocytes and thickness of lamina propria) measured later in life. Another objective of this study was to evaluate influence of supplemental Zn-Met in basal diet, which contained practical level of Zn, on indicators of gut immune responses as well as villus morphology in coccidiosis condition-because the coccidiosis is the prevalent pathogens of chickens and an economically important intestinal infection of poultry that lesions induced by pathogen are visible both macroscopically and microscopically.

MATERIALS AND METHODS

Experimental design and feeding patterns: This study was carried out in experimental farm of Isfahan University of Technology, Isfahan, Iran during September 2005 and April 2006. A total of 378 male and female seven day-old broiler chicks (Ross 308) were randomly assigned to 18 floor cages. Chicks were fed a basal diet supplemented with 0, 40 or 60 mg Zn kg^{-1} as Zn-Met which was administrated *ad libitum* (AL) or with feed restriction. The Zn content of basal diets (entire of ingredients except additive Zn-Met) was measured by atomic absorption spectrophotometer. The starter and grower diets contained 74 and 66 mg Zn kg^{-1}, respectively. The used Zn-Met (from Weifang Sunwin Chemical Co) contained 18.39% Zn and 43.51% methionine (analyzed by Co). Dietary methionine content differences between treatments (come from supplemented Zn-Met) were adjusted by additional D-L methionine. A complete random design was used in 3×2 factorial arrangement with 3 replicates during the 7 to 42 day period. The diets were formulated to meet NRC (1994) nutrient requirements (Table 1). Except for the duration of the FR, the chickens were fed AL. The FR program involved removing feeders on d 11, 13, 15 and 17 beginning at 08:00 to 08:00 am of the following day. The starter diet was given until day 21 and grower diet offered from 21 to day 42. Diets contained no coccidiostats.

Table 1: Ingredients and calculated nutrient content of diets

Ingredients (%)	Energy (KCal ME kg^{-1}) and calculated nutrient content (%) 7-21 day	21-42 day
Corn	55.56	64.23
Soybean meal (44%)	34.04	29.39
Fish meal	3.5	1.0
Fatty acid	3.5	2.0
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 (KCal ME/kg)	2991	2990
Crude protein	21.52	18.67
Lysine	1.25	0.93
Methionine	0.54	0.37
TSAA[D]	0.87	0.67
Calcium	0.93	0.81
Available phosphorus	0.42	0.37
Zn (mg kg^{-1})[E]	74	66

[A]Vitamin premix provides the following per kilogram of diet: 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 of diet: Mn, 55; Zn, 50; Fe, 50; Cu, 5; Se, 0.1; I, 1.5. [C]Variable amounts of zinc-methionine plus washed builders sand among the treatments. [D]TSAA: Total sulfur amino acids. [E]Zn content of basal diets (entire of ingredients except additive Zn-Met) was measured by atomic absorption spectrophotometer

Parasites and experimental infection: At days 28 of age, all birds were inoculated orally with a mixed culture of *Eimeria tenella* and *Eimeria acervulina* to simulate a coccidiosis challenge by 8.5×10^3 and 7.5×10^3 sporulated oocysts per chicken, respectively.

Tissue sampling and microscopic examination: On day 42, two chickens per pen were selected randomly and their 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 were examined finally by light microscope for the following: lesions score, 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.

Statistical analysis: Data were analyzed using the General Linear Model (GLM) procedures of SAS Institute (SAS, 1999) as a factorial experiment with the Zn-Met and feeding pattern as main effects. Comparisons among the means and interactions were made using Duncan's multiple range tests (Duncan, 1955) and Ls means procedure, respectively. The means differences were considered significant at a probability $p<0.05$.

RESULTS

Intestinal lesions were diminished significantly ($p<0.01$) by adding 40 or 60 mg kg^{-1} Zn-Met to diet comparing to unsupplemented group (Table 2). Especially in FR chickens it was more observable. The less intestinal lesions were observed by the more supplemental Zn-Met in diet, so that birds in treatment of 60 mg kg^{-1} Zn-Met with FR had lowest duodenal lesion score among the treatments. However, feeding pattern could not affect lesions condition in the intestine as main effect.

As shown in Table 2, the number of intraepithelial lymphocytes in duodenal sections did not differ significantly between FR and AL groups. But it was significantly ($p<0.05$) greater in the duodenal section of birds that dietary supplemented with 60 mg kg^{-1} Zn-Met comparing to the not supplemented group. The level of 40 mg kg^{-1} induced numerically but not statistically significant difference from 0 mg kg^{-1} level in this parameter.

The ratio of villus height/crypt depth was increased due to dietary supplementation with 60 mg kg^{-1} Zn-Met despite 40 mg kg^{-1} level of Zn-Met did not differ from 0 mg kg^{-1} level (Table 2). The highest V/C score was for treatment of 60 mg kg^{-1} Zn-Met with FR while lowest one was for not Zn-Met supplemented birds that consumed food AL. However, the ratio of V/C in duodenal sections was not influenced by feeding pattern.

There was no significant difference in villus surface area among the levels of additive Zn-Met. Birds in FR group had more expanded villus surface area than AL group (Table 2). The parameter of villus surface area included villus height, width and amount of folds on villus. In birds which had been assigned to FR group, duodenal plica were elongated with numerous folds on the villies and resulted in greater ($p<0.01$) surface area of the duodenum in these chickens (Fig. 1a, b). The thickness of duodenal lamina propria was not affected by level of additive Zn-Met but administration of FR led to highly significantly ($p<0.001$) thinner lamina propria of duodenum in chickens (Table 2).

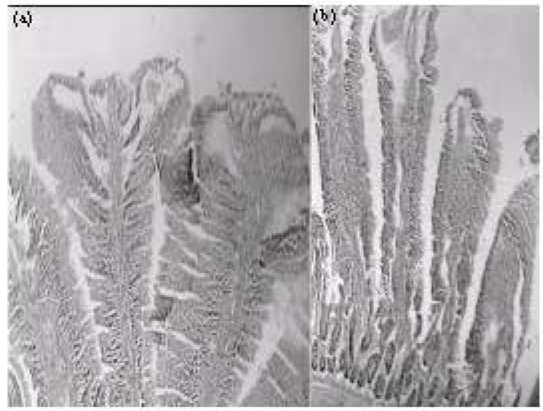

Fig. 1: Examples of villus surface area in two feeding pattern groups. (a) In Feed Restricted (FR) birds duodenal plica were elongated with numerous folds on the villies and resulted in larger surface area of the duodenum comparing to (b) *ad libitum* (AL) fed group

Table 2: Effects of zinc-metionine and feeding pattern on duodenal morphology

Treatments						
Zn-Met (mg kg^{-1})	Feeding pattern	Lesions score	Intraepithelial lymphocytes	V/C	Villus surface area	Lamina propria thickness
0	FR	3.59[A]	1.33	2.31	2.33	1.32
0	AL	3.11	2.18	1.83	1.65	3.51
40	FR	2.20	2.35	3.00	2.67	1.66
40	AL	2.29	2.66	2.16	2.50	3.00
60	FR	1.58	3.00	3.50	3.84	2.17
60	AL	2.50	2.84	3.17	1.34	3.48
SEM		0.456	0.466	0.505	0.523	0.553
Main effects Zn-Met (mg kg^{-1})						
0		3.35[a]	1.75[b]	2.08[b]	2.00[a]	2.42[a]
40		2.24[b]	2.50[ab]	2.58[ab]	2.58[a]	2.33[a]
60		2.04[b]	2.92[a]	3.33[a]	2.60[a]	2.83[a]
Feeding pattern						
FR		2.45[a]	2.22[a]	2.94[a]	2.94[a]	1.72[b]
AL		2.63[a]	2.55[a]	2.39[a]	1.83[b]	3.33[a]
SEM		0.222	0.208	0.225	0.263	0.281
Source (Probability>F)						
Zn-Met		**	*	*	NS	NS
Feeding pattern		NS	NS	NS	**	***
Zn-Met×pattern		NS	NS	NS	NS	NS

[A]Values are least square means of variables, which are made from comparative scores. [B]Zn-Met: zinc-methionine, FR: Feed restriction, AL: *ad libitum*, V/C: Villus height/Crypt depth ratio, SEM: Standard error of means. Values within variables with no common superscripts differ significantly ($p<0.05$). NS: Not Significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$

DISCUSSION

Within the intestinal mucosa, intestinal lymphocytes are present in two anatomic compartments, the epithelium and lamina propria (Befus *et al*., 1980) which these cells and T cells regulate mucosal immune responses. Intraepithelial lymphocytes have been demonstrated to secrete several cytokines, including interleukins, transforming growth factor and interferon-γ (Lundqvist *et al*., 1996; Mayer *et al*., 1991; Fan *et al*., 1998). In addition, 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). Therefore, this is almost obvious that theses cells are an important part of intestinal immune responses. According to presented results, dietary supplementation with Zn-Met in diets which contain enough level of Zn (according to NRC recommendations) improves this part of intestinal immune responses of chicken. Currently we do not have any knowledge of literature to support or refute this point.

Although, in this research, alternate day feeding early in life could not affect the ration of V/C later in life, but in other researches (Yamauchi *et al*., 1996; Shamoto *et al*., 1999; Tarachai and Yamauchi, 2000), in which birds had been served by several days feed withdrawal continually, the duodenal villus height and cellular proliferation within the crypt showed an immediate reduction during the fasting period and it was rapidly recovered by refeeding. But the base design of this project is completely different from ours which is cause of inconsistent results. However, as mentioned in results, alternate day feeding early in life expanded villus surface area later in life. 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 betaine (Kettunen *et al*., 2001; Klasing *et al*., 2002 especially in coccidiosis condition) and Zn deficiency in the diet (Southon *et al*., 1985). Indeed, in relation to villus height and crypt depth the important fact 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.

The villus surface area is a more reliable criterion for absorptive area than V/C ratio because it includes villus height, width and amount of folds on villus. Reduction of absorptive villus compartment, in coccidiosis condition and in general damage to the gut, including sloughing of the mucosa, reduces the absorptive surface and causes malabsorption (Ruff, 1978). Malabsorption is a typical symptom of coccidiosis (Scott *et al*., 1982; Girdhar *et al*., 2006). Since in this research dietary supplementation with Zn-Met increased ratio of V/C and administration of FR showed a potential to expand villus surface area, they could be benefit to reduce negative effects of coccidiosis on nutrient absorption and assist to more efficient utilization and absorption of nutrients. Our results (Hamidi and Pourreza, 2009) in performance parameter partially confirm this opinion. There was a significant interaction between Zn-Met and FR for feed conversion ratio. Feed restricted birds in 7 to 42 days period had lower feed conversion ratio when received 40 or 60 mg kg^{-1} Zn-Met comparing to those received no Zn-Met. On the other hand, reduction of lesion scores by dietary supplemental Zn-Met could be related to this issue, because the less gut lesions, the more absorptive area.

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 production (Macpherson and Harris, 2004). Although, in this study, administration of FR decreased thickness of lamina propria, as an indicator of gut health, but evaluation of morphological and histological parameters with other methods of FR administration, by different severity and various times after administration in all parts of intestine would reveals more knowledge of gut condition in FR birds. It is likely that the design of feed restriction administration cloud firmly affect results.

CONCLUSION

According to the effect of Zn-Met on intraepithelial lymphocytes and reduction of intestinal lesions it seems that preparing more available zinc in broilers diet improves immunocompetence of the gastrointestinal tract of birds. Moreover, morphological changes of intestine whose relate to absorptive compartment of intestine (V/C ratio and villus surface area), induced by dietary Zn-Met and FR administration, suggest that theses might have benefit to reduce adverse effects of coccidiosis on nutrients absorption.

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