



Effects of Methionine Deficiency on the B Lymphocyte and Immunoglobulins of Cecal Tonsil in Cobb Broilers

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ABSTRACT

The present study was conducted on one-day-old broilers to investigate the effects of methionine deficiency on B lymphocytes and immunoglobulins (slgA, IgA, IgG, IgM) in the cecal tonsil of Cobb broiler chicken. Methods including immunohistochemistry (IHC), western blot (WB), quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) and Enzyme-Linked Immuno Sorbent Assay (ELISA) were used, and the study lasted for 6 weeks. It was found that the IgA⁺ B lymphocytes mainly existed in the diffuse lymphoid tissues and lymphoid follicles of the cecal tonsil. The data indicated that the number of IgA⁺ B lymphocytes was decreased in the methionine deficiency group, and the WB and qRT-PCR results suggested that the protein and mRNA expression of CD19 were reduced in the methionine deficiency group. Besides, the contents of slgA, IgA, IgG and IgM determined by ELISA decreased in the methionine deficiency group. It could be concluded that methionine deficiency exerts significantly negative effects on the humoral immune function of the intestinal mucosal immunity.

INTRODUCTION

Methionine is an essential amino acid which possesses high nutritional value and important physiological functions, such as cellular growth and the development of animal (Mirzaaghatbar *et al.*, 2011; Yen *et al.*, 2002), detoxification (Kim *et al.*, 2006), antitumor or anticancer (Horvat *et al.*, 2006; Li *et al.*, 2009), resistance for coccidium infection (Rama Rao *et al.*, 2003) and synthesis of protein (Brosnan, 2006; Tesseraud *et al.*, 2011), etc. Moreover, methionine is closely related to the immune function of livestock and poultry, and is responsible for the regulation of cell function such as oxidation and reduction (Hoshi & Heinemann, 2010). Appropriate dietary methionine restriction improves colon tight junction barrier function, alters claudin expression pattern and improves epithelial barrier function (Ramalingam *et al.*, 2010).

Methionine deficiency has negative effects on immune organs including thymus, bursa of Fabricius, spleen (Wu *et al.*, 2012a; Wu *et al.*, 2012b; Wu *et al.*, 2013) and small intestine (oxidative stress and apoptosis) (Li *et al.*, 2018; Ruan *et al.*, 2018; Wu *et al.*, 2018a; Wu *et al.*, 2018b). The objective of this study is to determine the effects of methionine deficiency on the B lymphocytes and immunoglobulins in the cecal tonsil of broiler chicken by methods including IHC, WB, qRT-PCR and ELISA. Cecal tonsil (CT) is considered to be the largest lymphoid aggregate of avian gut-associated lymphoid tissue (GALT), CT, and elicits protective immune responses against bacterial or viral pathogens in the intestinal tract of avian species (Heidari *et al.*, 2014). Currently, few studies focus on the effects of methionine deficiency on the cecal tonsil in avian, so this research aims to investigate the role of methionine on the important intestinal immune organ – cecal tonsil.



MATERIALS AND METHODS

Birds, management and diets

The animal protocol used in this study was approved by the China West Normal University Animal Care Committee (Nanchong, China), and the study was carried out in accordance with the national standard on the protection of animals used for scientific purposes.

In total, 72 one-day-old male Cobb broiler chicken were used in this study. They were raised at the Research Laboratory of the Department of College of Life Science, China West Normal University in Nanchong (Sichuan, China). The birds were kept in pens on litter, in a cage with a controlled environment. The temperature and lighting programme aligned to the recommendations of national standard (Chinese, GB4925-2010). Briefly, the breeding temperature is controlled at 35-33 °C in the first week, 33-31 °C in the second week, 31-28 °C in the third week, and 28-24 °C after the fourth week. The relative humidity is between 60% and 70%, and the concentration of the CO₂ should not exceed 0.5%. In addition, plenty of light is also very important to the broiler chicken, on the first and second day, 24 hours of light is needed, and 23 hours from 3 to 6 days. After 7 days the time of light should be kept as 22 to 23 hours per day. It should be noted that the pens should be kept clean (cleaning up the stool in time), and the pens and the other appliances including the sink and feed bucket should be sterilized regularly in order to prevent bacterial contamination and keep the chicks healthy.

The chicken were randomly allotted to 2 groups by body weight (42±3g), with 6 replicates pens (n=6) per treatment group. In the methionine deficiency group, broilers were fed diet with methionine (DL-Met) supplementation, while in the control group, chickens were fed on the diet without methionine. All chicken were provided with water as well as aforementioned diets *ad libitum*. Twenty-four chicks from each small cage were euthanized at 14, 28 and 42 days of age, respectively (twelve in each group at each time point). Cecal tonsils were removed for observation and determination aseptically. Besides, mononuclear cells from the tissues were isolated for further detection.

The total Met content of diets corresponded to the level recommended by NRC (1994). The final concentrations of amino acids, including Met, in the diets were verified analytically (Table 1). In Met deficient diet, Met content was 0.26% for starter diet (1 to 21d), and was 0.28% for Met content in grower diet (22 to 42d). Besides, additional Met contents of 0.24% and 0.12% were added to Met deficient diet to

produce control diets (starter diet, Met 0.50%; grower diet, Met 0.40%) (Wu *et al.*, 2012a).

Table 1 – Components of the basal diets for broilers (%).

Ingredient, 1 %	Met deficient diet		Control diet	
	Starter diet 1 to 21d	Grower diet 22 to 42d	Starter diet 1 to 21d	Grower diet 22 to 42d
Ground yellow corn	56	59.5	56	59.5
Soybean meal	37	32.85	37	32.85
Soybean oil	3.66	4.7	3.66	4.7
Ground limestone	0.57	0.5	0.57	0.5
Dicalcium phosphate	1.8	1.6	1.8	1.6
Salt	0.3	0.3	0.3	0.3
Choline Chloride	0.1	0.1	0.1	0.1
DL-Met	0.0	0.0	0.24	0.12
Bentonite	0.24	0.12	0.0	0.0
Micronutrients ²	0.33	0.33	0.33	0.33
level, (%)				
Calculated Nutrients				
ME, MJ/kg	12.39	12.79	12.39	12.79
CP	21.17	19.72	21.17	19.72
Lys	1.19	1.08	1.19	1.08
Met	0.26	0.28	0.50	0.40
Met+Cys	0.62	0.62	0.86	0.74
Ca	0.85	0.77	0.85	0.77
Nonphytate P	0.44	0.40	0.44	0.40

¹Ingredient and nutrient composition are reported on as-fed basis.

²For the diet of d 1-21 and d 22-42, provided per kilogram of diet: vitamin A (all-trans retinol acetate), 12,500 IU; cholecalciferol, 2,500 IU; vitamin E (all-rac- α -tocopherol acetate), 18.75 IU; vitamin K (menadione Na bisulfate), 5.0 mg; thiamin (thiamin mononitrate), 2.5 mg; riboflavin, 7.5 mg; vitamin B₆, 5.0 mg; vitamin B₁₂, 0.0025 mg; pantothenate, 15 mg; niacin, 50 mg; folic acid, 1.25 mg; biotin, 0.12 mg; Cu (CuSO₄×5H₂O), 10 mg; Mn (MnSO₄×H₂O), 100 mg; Zn (ZnSO₄×7H₂O), 100 mg; Fe (FeSO₄×7H₂O), 100 mg; I (KI), 0.4 mg; Se (Na₂SeO₃), 0.2 mg.

Immunohistochemical examination (SABC) of the IgA⁺ B cells

IgA⁺ B cell expression in the cecal tonsil was detected immunohistochemically using the Strept Avidin-Biotin Complex (SABC) method, as described by Wu *et al.* (2018a). Briefly, cecal tonsils were fixed in neutralized 10% buffered formalin for 48 h and routinely processed for embedding in paraffin and sectioned to 4 μ m pieces. Tissue sections were dewaxing, dehydrated or rehydrated through xylene and a graded series of ethanol, washed in distilled water and phosphate buffer saline (PBS). After blocking with 3% endogenous peroxidase for 15 min, the slides were overlaid with 10% normal goat serum (NGS) in PBS at 37 °C. Sections were incubated in primary antibodies (diluted 1:100) (polyclonal mouse anti-chicken IgA heavy chains, Southern Biotech-8330-01) overnight at 4 °C (for negative tissue controls, PBS was used instead of the primary antibody), and incubated with 1% biotinylated secondary antibody goat anti-mouse IgG (ZSGB-BIO SP Kit) for 1 h at 37 °C. Then the pieces were incubated



with the HRP-streptavidin (ZSGB-BIO SP Kit) for 30 min at 37 °C. To visualize the immunoreaction, sections were immersed in diaminobenzidine hydrochloride (DAB). After hematoxylin counterstaining, the sections were examined with an Olympus AX 70 microscope (Olympus Corp., Tokyo, Japan) and photographed (magnification of $\times 400$). The IgA⁺ B cells (positive cells) were quantified by Image-Pro Plus 5.1 image analysis software. Each of the six chicken in each group were measured in five sections and each section was measured in five visions and averaged.

Detection of the CD19 protein expression by Western Blot

Cecal tonsil proteins were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions. Proteins (3 μ l of sample at 1/10 dilution) were separated on polyacrylamide gels containing a stacking gel of 4% and a running gel of 10% and transferred to Immobilon-P (polyvinylidene difluoride (PVDF) millipore membranes (transfer buffer: 12.5 mM Tris, 98 mM glycine and 10% methanol) at the transfer conditions of 150 V for 1.5 h at 4 °C. Unstained markers for molecular weight from Sigma were included in all gels. After the transfer, the PVDF membrane was stained with 0.1% Red Ponceau with the aim of separating the lanes containing the molecular weight markers. Then the transfer membranes without molecular weights were washed with 150 mM PBS, pH 7.2 plus 0.05% Tween-20 (PBS-T) and incubated in blocking buffer (PBS-T plus 5% non-fat dry milk) for 1 h. Next, the membranes were incubated with anti-CD19 antibody (ab37808, Abcam, England) conjugated with peroxidase (1/1000 dilution, diluted with PBS-T) at 4 °C overnight. Three washes with PBS-T were performed after each step (30 min). Then membranes were incubated with the secondary antibody (1/5000 dilution, diluted with PBS-T) at room temperature for 1 h, and then washed again. Once the process was finished, the blots were dried and immediately scanned. Protein bands were quantified using 1D image analysis software (Scion Image for Windows, Scion Corporation).

Detection of the CD19 mRNA expression by qRT-PCR

The cecal tonsils were obtained from six broilers in two groups at 14, 28, and 42 days of age. RNA was isolated from cecal tonsils using the RNA isolation kit (9108/9109, Takara, Japan). RNA was extracted and purified as described by Wu *et al.* (2014). RNA was quantified using the NanoDrop

spectrophotometer (NanoDrop Technologies) and 0.5 μ g reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed using an iCycler iQ Real-Time PCR detection system using iQ SYBR Green Supermix (BioRad). Expression level of target gene (CD19) was quantified and the expression of the β -actin housekeeping gene served as control. Gene expression values of the control group were used each week for gene expression calibration, respectively. With 2^{- Δ ACT} assay, the results were analyzed.

Determination of the immunoglobulins (IgG, IgA, IgM and sIgA) contents in the cecal tonsil by ELISA

The cecal tonsil tissues were collected and stored at -80 °C prior to the measurement of ELISA parameters. The immunoglobulin contents were determined using ELISA kit (MM-0912) as described by Wu *et al.* (2012b), and the result were calculated by the standard curve and were expressed as nanogram per millilitre. Briefly, 40 μ l of the samples were diluted and added to Standard (blank) wells and testing wells, and were incubated at 37 °C for 30 min. Then, the wells were washed with washing buffer 5 times and dried by pat. Then 50 μ l HRP-Conjugate reagent was added to each well, except blank well, and incubation and wash were conducted as the operation abovementioned. After that, Chromogen Solution A 50 μ l and Chromogen Solution B were added to each well, and preserved evading the light for 15 min at 37 °C. The absorbance was read at 450nm after adding Stop Solution. Sample OD value was calculated according to the standard curve, and then the sample density was calculated based on the OD value.

Statistical analysis

Results were expressed as mean values \pm standard deviation ($M \pm SD$). Data were analyzed using Independent-Samples T test followed by Post Hoc, Tukey's HSD test. All Statistical analysis was performed using SPSS (SPSS for Windows, version 16.0, SPSS Inc, Chicago, Illinois). Differences were considered statistically significant when the calculated p-value was less than 0.05 and $p < 0.01$ was considered highly significant.

RESULTS

Changes in IgA⁺ B cells by Immunohistochemical examination (SABC)

The results of immunohistochemical examination showed that IgA⁺ B positive cells were stained brown,



and distributed in the diffuse lymphoid tissues and lymphoid follicles of the cecal tonsil as shown in Figure 1 a-f. The quantity of IgA⁺ B cells in the methionine deficiency group was significantly lower ($p < 0.01$) than that in the control group at 28 and 42 days of age, while no significant changes were observed at 14 days of age, which has been presented in Fig. 2.

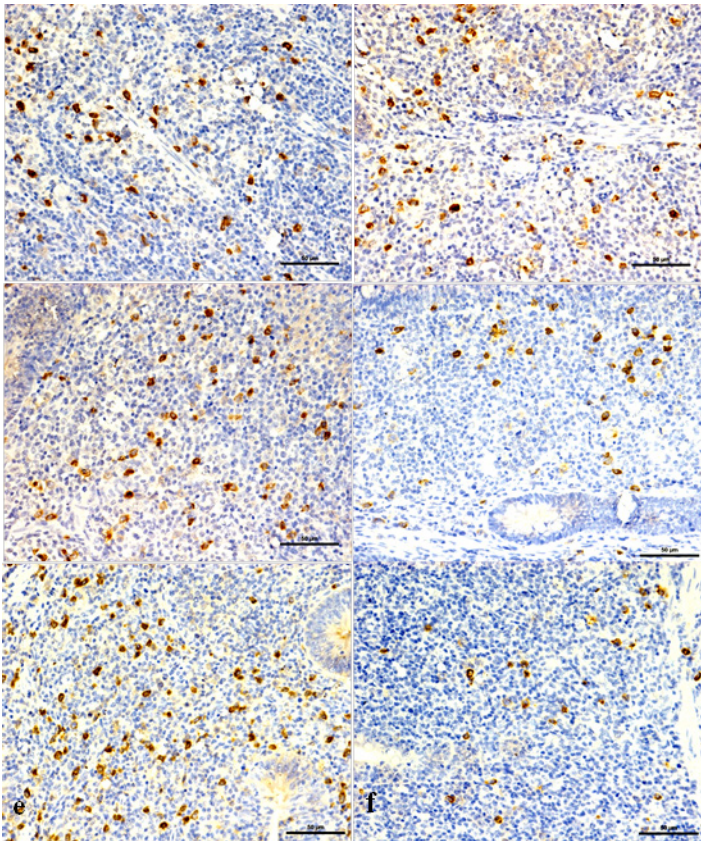


Figure 1 – IgA⁺ B cells in the diffuse lymphoid tissues and lymphoid follicles of the cecal tonsil

a. The IgA⁺ B cells in the control group (bar=50 μm) at 14 days of age. b. The IgA⁺ B cells in the Met deficiency group at 14 days of age. There is no significant change (bar=50 μm). c. The IgA⁺ B cells in the control group at 28 days of age. d. The quantities of IgA⁺ B cells significantly decreased in the Met deficiency group at 28 days of age (bar=50 μm). e. The IgA⁺ B cells in the control group at 42 days of age. f. The quantities IgA⁺ B cells significantly decreased in the Met deficiency group at 42 days of age (bar=50 μm).

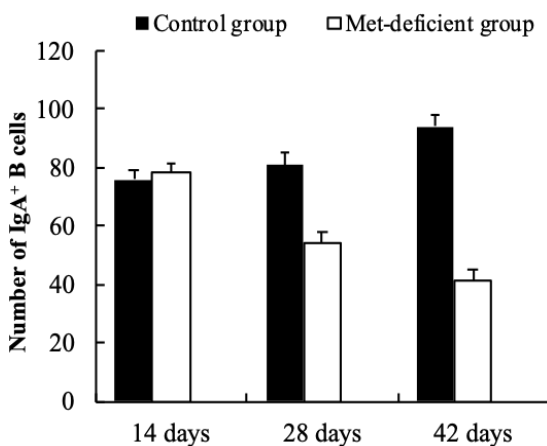


Figure 2 – The number of IgA⁺ B cells in cecal tonsil

CD19 protein and mRNA expression level in cecal tonsil

CD19 protein and mRNA expression levels in the cecal tonsil of methionine deficiency group were significantly decreased ($p < 0.05$ or $p < 0.01$) at 28 and 42 days of age when compared with the control group (Fig. 3 a, b and c).

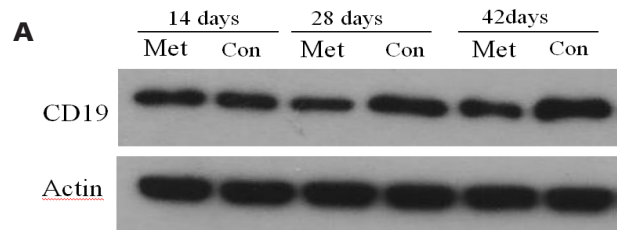


Figure 3 CD19 protein expression in cecal tonsil

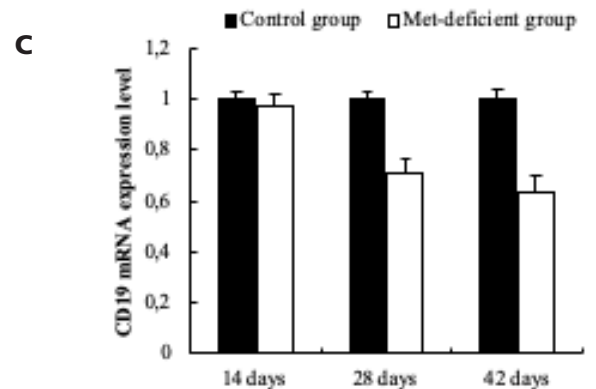
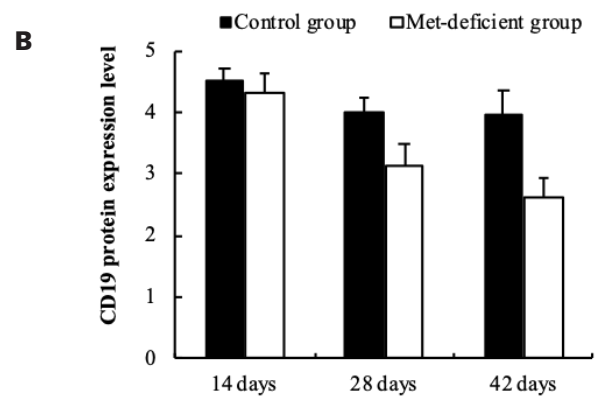


Figure 3 – a. CD19 protein expression in cecal tonsil; b. Relative expression of CD19 protein in cecal tonsil; c. CD19 mRNA expression level.

Change of the sIgA, IgA, IgG and IgM contents (μg/ml) in cecal Tonsil

As shown in Fig. 4 a-d, the sIgA contents in the cecal tonsil of methionine deficiency group were significantly lower ($p < 0.05$ or $p < 0.01$) than in the control group at 14, 28 and 42 days of age. And the IgA, IgG and IgM contents showed the similar trend as they all significantly decreased ($p < 0.05$ or $p < 0.01$) at 28 and 42 days of age.

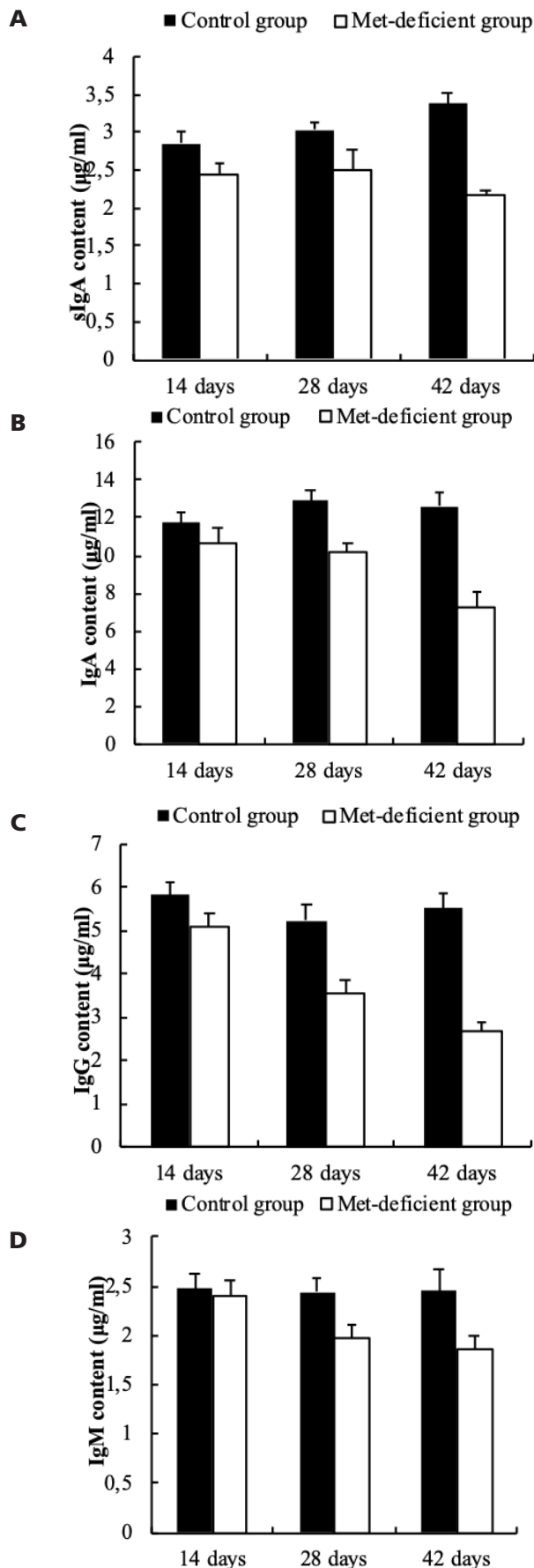


Figure 4 – Change of the sIgA, IgA, IgG and IgM contents (µg/ml) in cecal Tonsil

DISCUSSION

In this study, the changes of B lymphocytes count and the immunoglobulins contents including sIgA, IgA, IgG and IgM in the cecal tonsil induced by methionine deficiency were observed. It is well known that the cecal tonsil is the major lymphoid tissue in avian cecum which not only participates in the antibody production but also is an important component in the mucosal immune system. The mucosal immune system in the cecal tonsil provides antigen-specific protection through B cells/antibodies and mucosal T cells, among which the B cells or the produced antibodies compose the humoral immunity (Clem & AngelaS, 2011). B cells in the cecal tonsil can migrate into the lamina propria and develop into mature immunoglobulin-producing plasma cells (Fagarasan *et al.*, 2011). In the present research, we found that the methionine deficiency reduced the population of B lymphocytes, the protein and mRNA expression of the CD19, and the immunoglobulin contents including sIgA, IgA, IgG and IgM.

IgG (IgY), IgM and IgA are the major immunoglobulin classes in chicken (Mockett, 1986). IgA or secretory IgA (sIgA) is critical in protecting the mucosal surface against pathogenic microorganism (such as the toxins, viruses or bacteria) (Mockett, 1986). In addition, sIgA plays important roles in the maintenance of mucosal homeostasis and the development of systemic immunity and may also determine the composition of the intestinal microbiota (Lammers *et al.*, 2010). The decrease of IgA or sIgA indicates that the protective role of the intestinal mucosal is impaired due to methionine deficiency. Furthermore, there is a close relationship between B cells (IgA⁺ B cells) and the humoral immune response, which implies that the reduction of B cell population, sIgA and IgA production will eventually impact the humoral immune function in the mucosal immunity of the cecal tonsil in broilers.

Chicken IgM is homologous to mammalian counterpart structurally and functionally (Perez-Carbajal *et al.*, 2010), which is the first antibody generated during the primary antibody response. IgM is also the major class of immunoglobulin expressed on the surface of chicken B lymphocytes (Boes, 2000). In the present study, the content of IgM in the cecal tonsil was decreased in the methonine deficiency group, which indicates that dietary methonine deficiency can impair primary immune response and has negative effects on B lymphocytes.

IgG, which is secreted by B lymphocytes and was generated mainly in the secondary antibody



responses, behaves like the homologue of mammalian IgG (Shimizu *et al.*, 1992). IgG is the main antibody isotype found in blood and extracellular fluid, so it plays important roles in the protection of the body tissues (Hirano *et al.*, 1986). Our present study showed that IgG contents decreased in the cecal tonsil of chicken fed on daily methionine deficiency diet. Thus, we speculated that dietary methionine deficiency could impact the clearance of the pathogens and the immune response. Our previous study also reported that the methionine deficiency significantly reduced the synthesis of IgG antibody in the duodenum and jejunum (Wu *et al.*, 2018a).

In summary, our study suggested that methionine deficiency has negative effects on the B lymphocytes and contents of immunoglobulins including sIgA, IgA, IgG and IgM in the cecal tonsil of broiler chicken. As a result, methionine might exert significant effect on the humoral immune function of intestinal mucosal immunity. This study provides the new experimental information and is pivotal for studying the effects of methionine on the intestinal function in human and other animals in the future.

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CONFLICT OF INTEREST

The authors declare no competing financial interest. The use of chickens and all experimental procedures were approved by China West Normal University Animal Care and Use Committee.

REFERENCES

- Boes M. Role of natural and immune IgM antibodies in immune responses. *Molecular Immunology* 2000;37(18):1141-1149.
- Brosnan JT, Brosnan ME. The sulfur-containing amino acids: an overview. *Journal of Nutrition* 2006;136 (6 Suppl) :1636S-1640S.
- Clem AS. Fundamentals of vaccine immunology. *Journal of Global Infectious Diseases* 2011;3(1):73-78.
- Fagarasan S, Kinoshita K, Muramatsu M, Ikuta K, Honjo T. In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature* 2011;413(6856):639-643.
- Heidari M, Fitzgerald SD, Zhang H. Marek's disease virus-induced transient cecal tonsil atrophy. *Avian Diseases* 2014;58(2):262-270.
- Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, *et al.* Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* 1986;324(6092):73-76.
- Horvat S, Mlinaric-Majerski K, Glavas-Obrovac L, Jakas A, Veljkovic J, Marczi S, *et al.* Tumor-cell targeted methionine-enkephalin analogues containing unnatural amino acids: design, synthesis, and in vitro antitumor activity. *Journal of Medicinal Chemistry* 2006;49(11):3136-3142.
- Hoshi T, Heinemann SH. Regulation of cell function by methionine oxidation and reduction. *Journal of Physiology* 2010;531(1):1-11.
- Kim WK, Froelich CA, Patterson PH, Ricke SC. The potential to reduce poultry nitrogen emissions with dietary methionine or methionine analogues supplementation. *World's Poultry Science Journal* 2006;62(2):338-353.
- Lammers A, Wieland WH, Kruijt L, Jansma A, Straetmans T, Schots A, *et al.* Successive immunoglobulin and cytokine expression in the small intestine of juvenile chicken. *Developmental and Comparative Immunology* 2010;34(12):1254-1262.
- Li C, Li Z, Sletten E, Arnesano F, Losacco M, Natile G, *et al.* Methionine can favor DNA platination by trans-coordinated platinum antitumor drugs. *Angewandte Chemie* 2009;48(15):8497-8500.
- Li LG, Ruan T, Peng X, Wu BY. Immunosuppression of methionine deficiency on the number of IgA⁺ B cells and sIgA content in the cecal tonsil of broiler chicken. *Indian Veterinary Journal* 2018;95 (2):26-28.
- Mirzaaghatabar F, Saki AA, Zamani P, Aliarabi H, Matin HRH. Effect of different levels of diet methionine and metabolisable energy on broiler performance and immune system. *Food and Agricultural Immunology* 2011;22(2):93-103.
- Mockett APA. Monoclonal antibodies used to isolate IgM from chicken bile and avian sera and to detect specific IgM in chicken sera. *Avian Pathology* 1986;15(3):337-348.
- Perez-Carbajal C, Caldwell D, Farnell M, Stringfellow K, Pohl S, Casco G, *et al.* Immune response of broiler chickens fed different levels of arginine and vitamin E to a coccidiosis vaccine and *Eimeria* challenge. *Poultry Science* 2010;89(9):1870-1877.
- Rama Rao SV, Prahara JNK, Panda AK, Reddy MR. Interaction between genotype and dietary concentrations of methionine for immune function in commercial broilers. *British Poultry Science* 2003;44(1):104-112.
- Ramalingam A, Wang X, Gabello M, Valenzano MC, Mullin JM. Dietary methionine restriction improves colon tight junction barrier function and alters claudin expression pattern. *AJP Cell Physiology* 2010;299(5):C1028-1035.
- Ruan T, Li LJ, Lyu YN, Lyu, Luo Q, Wu BY. Effect of methionine deficiency on oxidative stress and apoptosis in the small intestine of broilers. *Acta Veterinaria Hungarica* 2018;66(1):52-65.
- Shimizu M, Nagashima H, Sano K, Hashimoto K, Ozeki M, Tsuda K, *et al.* Molecular stability of chicken and rabbit immunoglobulin G. *Journal of the Agricultural Chemical Society of Japan* 1992;56(2):270-274.
- Tesseraud S, Everaert N, Ezzine SB, Collin A, Métayer-Coustard S, Berri C. Manipulating tissue metabolism by amino acids. *World's Poultry Science Journal* 2011;67(2):243-252.
- Wu BY, Cui HM, Peng X, Fang J, Cui W, Liu XD. Pathology of spleen in chickens fed on a diet deficient in methionine. *Health* 2012a;4(1):32-38.
- Wu BY, Cui HM, Peng X, Fang J, Cui W, Liu XD. Effect of methionine deficiency on the thymus and the subsets and proliferation of peripheral blood t-cell, and serum il-2 contents in broilers. *Journal of Integrative Agriculture* 2012b;11(6):1009-1019.



Wu BY, Cui HM, Peng X, Fang J, Cui W, Liu XD. Pathology of bursae of Fabricius in methionine-deficient broiler chickens. *Nutrients* 2013;5(3):877-886.

Wu BY, Li LY, Ruan T, Peng X. Effect of methionine deficiency on duodenal and jejunal IgA⁺ B cell count and immunoglobulin level of broilers. *Iranian Journal of Veterinary Research* 2018a;19(3):165-171.

Wu BY, Zhu M, Ruan T, Li LJ, Lyu YN. Oxidative stress, apoptosis and abnormal expression of apoptotic protein and gene and cell cycle arrest in the cecal tonsil of broilers induced by dietary methionine deficiency. *Research in Veterinary Science* 2018b;121:65-75.

Wu BY, Cui HM, Peng X, Fang J, Zuo ZC, Deng JL, et al. Dietary nickel chloride induces oxidative stress, apoptosis and alters Bax/Bcl-2 and caspase-3 mRNA expression in the cecal tonsil of broilers. *Food and Chemical Toxicology* 2014;63:18-29.

Yen CE, Mar MH, Craciunescu CN, Edwards LJ, Zeisel SH. Deficiency in methionine, tryptophan, isoleucine, or choline induces apoptosis in cultured cells. *The Journal of Nutrition* 2002;132(7):1840-1847.

