Effect of in ovo feeding of folic acid on the folate metabolism, immune function and epigenetic modification of immune effector molecules of broiler

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Abstract

This study was conducted to investigate the effect of in ovo feeding (IOF) of folic acid on the folate metabolism, immune function and the involved epigenetic modification of broilers. A total of 400 (Cobb) hatching eggs were randomly divided into four groups (0, 50, 100 and 150 µg injection of folic acid at embryonic age 11 d), and chicks hatched from each treatment were randomly divided into six replicates with 12 broilers/replicate after incubation. The results indicated that, in ovo, 100- and 150-µg folic acid injections improved the hatchability. The average daily gain and feed conversion ratio increased in the 150-µg group during the late growth stage. Simultaneously, in the 100- and 150-µg groups, an increase was observed in hepatic folate content and the expression of methylenetetrahydrofolate reductase (d1 and d2) and methionine synthase reductase (d2). IgG and IgM concentrations, as well as plasma lysozyme activity of broilers, showed a marked increase along with increasing folic acid levels. The splenic expression levels of IL-2 and IL-4 were up-regulated, whereas that of IL-6 was down-regulated, in the 100- and 150-µg folic acid treatment groups. In addition, histone methylation in IL-2 and IL-4 promoters exhibited an enrichment of H3K4me2 but a loss of H3K9me2 with the increased amount of folic acid additive. In contrast, a decrease in H3K4me2 and an increase in H3K9me2 were observed in the IL-6 promoter in folic acid treatments. Furthermore, in ovo, the 150-µg folic acid injection improved the chromatin tightness of the IL-2 and IL-4 promoter regions. Our findings suggest that IOF of 150 µg of folic acid can improve the growth performance and folate metabolism of broilers, and enhance the relationship between immune function and epigenetic regulation of immune genes, which are involved with the alterations in chromatin conformation and histone methylation in their promoters.

Key words: Folic acid; Folate metabolism; Immune function; Epigenetic modifications; Broilers

It is critical for the establishment of the epigenome that during gametogenesis and embryogenesis the epigenome experience elimination and reconstruction twice, respectively(11). Consequently, these two periods, which are vulnerable to the environment (such as nutrition, climate and so on), are the focus of epigenetics studies(2,3). Unlike in mammals, in poultry, embryonic development is mainly separated from the matrix. It is therefore difficult to estimate the effect of nutrients on embryogenesis if nutrients exhibit little or no accumulation in eggs. Currently, in ovo feeding (IOF) is gradually operational(4,5) and it can thus be used to evaluate the effect of specific nutrients on epigenome reprogramming, providing an effective approach to the study of nutri-epigenetics.

Folic acid, a water-soluble B-complex vitamin, also known as pteroylglutamic acid, has a critical role in one-carbon metabolism(6,7). Similarly, folic acid is a central co-enzyme for both DNA methylation and DNA synthesis(8). Methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20), a key enzyme in folate metabolism, has a key role in both biological methylation and nucleotide synthesis, balancing these two pathways to preserve normal homoeostasis(7,9). In addition, methionine synthase reductase (MTRR), another pivotal enzyme, is essential for folate and homocysteine metabolism(10).

As a methyl group, folate functions as a ground substance for the formation of methionine, which can be converted to S-adenosylmethionine (SAM)(9). In the methionine metabolic cycle, SAM is further converted to S-adenosylhomocysteine, thereby donating the methyl group for DNA and protein methylation, and finally changing chromatin conformation(2). Consequently, folate supplementation may modulate gene expression by modifying epigenetic marks such as histone methylation and chromatin conformation.

Folate insufficiency is associated with the clinical syndrome including CVD and intra-uterine growth retardation(11,12), whereas folate supplementation may result in decreased abnormal embryologic development by increasing fetal folate concentrations and supporting normal embryonic DNA synthesis during gestation(13). Folate, as a methyl donor, is able to reverse the epigenetic marks and thereby recover the metabolic disorders in offspring caused by prenatal or neonatal...
abnormal development.\(^\text{(2,14,15)}\) It has also been observed that folate supplementation can enhance the immunity of domestic animals.\(^\text{(16–18)}\) However, although folate is fundamental for embryonic and fetal development, whether IOF of folic acid can influence the growth performance, folate metabolism and immune function of broilers has not been well investigated.

The present experiment was therefore undertaken to evaluate the effects of folic acid injection during incubation on the growth performance and immune function of broilers. Simultaneously, to explore the potential epigenetic mechanisms underlying such effects, we studied the splenic expression of conformation and histone methylation on their promoters. The information obtained in this study can therefore be useful for understanding the role of folate on growth and immunity adjustment in poultry.

**Methods**

**Animals and sampling**

**Incubation.** A total of 400 fertilised broiler (Cobb 500) eggs were selected with an average weight of 65.7 g. Eggs were randomly assigned to four groups – 0, 50, 100 and 150 µg of folic acid – and each group was uniformly assigned to five incubator trays. The microcomputer automatic incubator (9TV-2A; Beijing LanTianJiao Electronic Technology Co., Ltd) was calibrated before hatching. The inner temperature of the incubator was controlled at 38.0–38.2°C from d1 to 10, at 37.8–38.0°C from d11 to 18 and at 37.5–37.8°C from d19 to 21. The humidity was maintained at 45–65%. A 270° overturn of the eggs was continued for 3 min every 2 h until d19. On d5 and 10, all eggs were candled, after which infertile and dead eggs were removed. Levels of folic acid were injected into the yolk sac at embryonic age 11 d, and the injection volume of each egg was 0.1 ml. The incubation period was 21 d.

The hatchability of fertilised eggs (%) was calculated as follows: (the number of hatchlings/the number of fertilised eggs)×100.

**Feeding.** Each treatment was allocated to six replicates with 12 broilers/replicate after incubation. The composition of the experimental diet is shown in Table 1. All birds were placed in three-layer wired battery cages and housed in an environmentally controlled room maintained at 32–34°C for the first week and then reduced by 2–3°C/week. The relative humidity was set at 50% throughout the study, and the lighting programme was 23 h of light for the first 2 weeks and 20 h thereafter. Birds were given free access to commercial diet and water. Feeds were offered as pellets in the whole phase, and a disc automatic feeding system was used to avoid feed spillage. The feeding period lasted for 42 d.

**Sampling.** On d1, 21 and 42, birds were first weighed in bulk, and then one bird approximative to the average body weight (BW) was selected from each replicate and weighed after fasting for 12 h. Blood samples were taken from the wing vein, and the plasma samples were obtained by centrifugation at 2500 rpm for 10 min at 4°C and then frozen at −20°C for analysis. After the blood sampling, broilers were electrically stunned and executed by exsanguination and necropsied immediately. Spleen and liver were removed within 15 min postmortem, snap-frozen in liquid N₂ and stored at −80°C until further analysis.

The experimental protocol was in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised 2004) and approved by the Institutional Animal Care and Use Committee (College of Animal Science and Technology, Northwest A&F University, China).

**Measurement of growth performance**

The BW was recorded for each replicate on d1, 21 and 42; concomitantly, the feed intake was recorded. BW, average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were then calculated.

**Determination of hepatic folate content and plasma lysozyme activity, IgG and IgM concentration**

A microbial method was used to determine the hepatic folate content.\(^\text{(19)}\) Briefly, approximately 200 mg of liver tissue was homogenised and diluted to 10 ml with phosphate buffer solution (PBS) (0.02 mol/l, pH 7.2) containing 50 mg of ascorbate. An aliquot of 0.2 ml of homogenate was autoclaved with buffer for 25 min, and then 1 ml of chicken pancreas extract (6 mg/ml) was added (Difco), further diluted to 10 ml with PBS and incubated overnight (37°C). After centrifugation (3000 rpm, 10 min), a further 5-fold dilution was made for the supernatant before addition to the assay tubes. The folate content in the culture medium has a

### Table 1. Ingredients and nutrient composition of broiler diets on fed basis\(^\text{(52)}\)

<table>
<thead>
<tr>
<th>Items</th>
<th>1–21 d</th>
<th>22–42 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize</td>
<td>57.80</td>
<td>62.80</td>
</tr>
<tr>
<td>Soyabeen meal (43 % CP)</td>
<td>34.60</td>
<td>29.70</td>
</tr>
<tr>
<td>Soyabeen oil</td>
<td>3.00</td>
<td>4.00</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.30</td>
<td>0.60</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>1.60</td>
<td>1.30</td>
</tr>
<tr>
<td>Salt</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>α-Met</td>
<td>0.20</td>
<td>0.10</td>
</tr>
<tr>
<td>L-Lys</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Premix*</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Calculation of nutrients (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME (MJ/kg)</td>
<td>12.33</td>
<td>12.92</td>
</tr>
<tr>
<td>CP</td>
<td>20.70</td>
<td>19.00</td>
</tr>
<tr>
<td>Ca</td>
<td>1.00</td>
<td>0.85</td>
</tr>
<tr>
<td>Available P</td>
<td>0.44</td>
<td>0.40</td>
</tr>
<tr>
<td>Total Lys</td>
<td>1.20</td>
<td>1.05</td>
</tr>
<tr>
<td>Total Met</td>
<td>0.50</td>
<td>0.38</td>
</tr>
<tr>
<td>Total Met + Cys</td>
<td>0.84</td>
<td>0.68</td>
</tr>
</tbody>
</table>

CP, crude protein; ME, metabolisable energy.

* Premix provided per kg of feed: vitamin A, 8000 IU; vitamin D₃, 2500 IU; vitamin K₃, 2.65 mg; vitamin B₁, 2 mg; vitamin B₂, 6 mg; vitamin B₁₂, 0.025 mg; vitamin B₆, 0.025 mg; vitamin E, 30 IU; biotin, 0.0325 mg; pantothenic acid, 12 mg; niacin, 50 mg; Cu, 8 mg; Zn, 75 mg; Fe, 80 mg; Mn, 100 mg; Se, 0.15 mg; I, 0.35 mg.  

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direct relationship with the growth and propagation of *Lactobacillus casei* ATCC 7469, and bacterial multiplication is computed by the optical density (OD) value. After an overnight incubation at 37°C, the turbidity was measured at 540 nm. The folate contents were calculated and expressed as µg/g of liver tissue.

The lysozyme activity and the concentration of IgG and IgM in plasma were measured using commercially available kits (A050-1, E026 and E025, respectively; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer’s instructions.

**Real-time PCR for mRNA quantification**

The total RNA was extracted from tissue samples using a total RNA extraction kit (9767; Takara) according to the manufacturer’s protocol. Total RNA was quantified using the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific) with the OD value at 260 nm, and the purity was assessed by determining the OD260:OD280 ratio and formaldehyde-agarose gel electrophoresis. The complementary DNA (cDNA) was synthesised with a PrimeScript® RT reagent Kit (Takara) at 37°C for 30 s, 72°C for 30 s; and 72°C for 5 min. All samples were run in triplicate, and the analytical method used for MNase-quantitative PCR data was previously reported (21,22).

**Chromatin immunoprecipitation assay for histone methylation**

DNA combined with the dimethylation of histone H3 lysine 4 (H3K4me2) or H3K9me2 was prepared using the EpiQuik™ Tissue Methyl-Histone H4 (H3K9) chromatin immunoprecipitation (ChIP) kit (P2009 and P2008, respectively; Epigentek). Next, quantitative PCR was performed to determine the accessibility to promoters of genes (II-2, IL-4 and IL-6). A reaction system (25 µl) was as follows: Quantitect (2 µl), 10 µl primer mix (5 µl), 2 µl DNA, 2 µl; and double-distilled water, 11 µl. Primer sequences used were all obtained from GenBank and are listed in Table 2. The reaction protocol was as follows: 95°C for 15 min, followed by 10 cycles of 95°C for 15 s, 70°C for 30 s and 72°C for 45 s, and then followed by another 35 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 45 s. All samples were run in triplicate, and the analytical method used for MNase-quantitative PCR data was previously reported (21,22).

**Micrococcal nuclease quantitative PCR assay for promoter conformation**

Briefly, the cell nuclei were extracted from spleen tissue using a nucleus extraction kit (CN1100, Solarbio) first, and then a suitable dose of micrococcal nuclease (MNase) (N3755, Sigma) was selected to digest these cell nuclei. Next, the histone was digested by proteinase K to obtain DNA. Last, the PCR was conducted with a 20-µl mixture system containing the following: 10 µl of SYBR® Premix Ex Taq II (2X), 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), 1 µl of DNA and 7 µl of double-distilled water. The primers used are listed in Table 2. The PCR cycle conditions were set as follows: 95°C for 15 min, followed by 10 cycles of 95°C for 15 s, 70°C for 30 s and 72°C for 45 s, and then followed by another 35 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 45 s. All samples were run in triplicate, and the analytical method used for MNase-quantitative PCR data was previously reported (21,22).

**Table 2. Primer sequences for quantitative real-time PCR analysis**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Accession number</th>
<th>Primer sequence (5′-3′)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>NM_205518</td>
<td>F: ATGTCCACCCGCAAATGCTTC 113</td>
<td>R: AAATAAAGCCATGCCAATCTCGTC</td>
</tr>
<tr>
<td>For RT-PCR</td>
<td>MTHFR</td>
<td>XM_417645.3</td>
<td>F: CGAACCCATCAAGGATAACGA 141</td>
</tr>
<tr>
<td></td>
<td>MTRR</td>
<td>XM_004935130.1</td>
<td>F: TTCTATTCAATCCTGGTGCTCTGGA 71</td>
</tr>
<tr>
<td></td>
<td>IL-2</td>
<td>NW_003763741.1</td>
<td>F: GCTATGACTACAGCTCCTGAAC 138</td>
</tr>
<tr>
<td></td>
<td>IL-4</td>
<td>NW_003763914.1</td>
<td>F: AACATGCGTCAGTTGGTGTGTAGAG 98</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>NW_003763664.1</td>
<td>F: AAATCCCTCCTCGGAACTAT 106</td>
</tr>
<tr>
<td>For MNase-qPCR and ChIP-qPCR</td>
<td>IL-2</td>
<td>NW_003763741.1</td>
<td>F: TTCCGAGGTGGCTGAGACAG 211</td>
</tr>
<tr>
<td></td>
<td>IL-4</td>
<td>NW_003763914.1</td>
<td>F: GAGGGACAGGTGGGAGAG 207</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>NW_003763664.1</td>
<td>F: GGTATTTGCTGCTTTTCTCGGA 203</td>
</tr>
</tbody>
</table>

**Notes:** MTHFR, methylenetetrahydrofolate reductase; MTRR, methionine synthase reductase; MNase, micrococcal nuclease; ChIP, chromatin immunoprecipitation; qPCR, quantitative PCR.
Compared with the control group, 50 \mu g of folic acid increased (P=0.025) the hatchability and an increasing trend (P=0.051) was found in the 150-\mu g folic acid group.

Although there were no significant differences on ADFI in the different feeding periods, an increasing trend (P=0.061) was observed during d21–42 with the increased amount of folic acid additive (Table 3). During d21–42 and d1–42, ADG showed an increase (P<0.05) and FCR had a significant decrease (P<0.05) in treatment groups with 100 and 150 \mu g of folic acid. It was interesting that the injection of a higher amount of folic acid (150 \mu g) gave rise to an improved BW on d21 (P<0.05), which was not observed on d42.

Folate content in the liver

Compared with the control group, in vivo injection of 50, 100 and 150 \mu g of folic acid all increased the hepatic content of folic acid on d21 (P<0.05), and an increase was also observed in the 100- and 150-\mu g groups on d42 (P<0.05) (Table 4). The expression of MTHFR was greater in the 150-\mu g group on d1 and 42 and in the 50-\mu g group on d1 (P<0.05)
Lysozyme activity, and the concentration of IgG and IgM in plasma

The lysozyme activity in plasma exhibited an increase \( (P<0.05) \) with increasing folic acid concentrations on d1, 21 and 42 (Table 5). Compared with the saline group, the IgG concentration was substantially improved \( (P<0.01) \) in the 100- and 150-\( \mu g \) folic acid groups on d1, 21 and 42. Similarly, on d21 and 42, folate treatments (50, 100 and 150-\( \mu g \)) all showed an increase \( (P<0.05) \) in the plasma content of IgM; however, no effect was found on d1.

Expression of IL-2, IL-4 and IL-6 in the spleen

In ovo, 150-\( \mu g \) folic acid injection on embryonic age 11 d increased \( (P<0.05) \) the expression of IL-2 while decreasing \( (P<0.05) \) the expression of IL-6 on d21 and 42 (Fig. 2(b) and (c)) in the spleen. The expression of IL-4 exhibited an increase \( (P<0.05) \) in the 100- and 150-\( \mu g \) folic acid treatment groups (Fig. 2(a), (b) and (c)).

Chromatin immunoprecipitation quantitative PCR for the histone methylation of IL-2, IL-4 and IL-6 promoters in the spleen

On both d21 and 42, more enriched H3K4me2 mark \( (P<0.05; \text{Fig. } 3(a)) \) was detected in the IL-2 promoter in the spleens of broilers hatched to 150-\( \mu g \) folic acid-injected eggs. Meanwhile, in ovo, 100- and 150-\( \mu g \) folic acid injections resulted in an enrichment \( (P<0.05; \text{Fig. } 3(b)) \) of H3K4me2 mark in the IL-4 promoter. In contrast, the levels of H3K4me2 in the IL-6 promoter exhibited a decrease \( (P<0.05; \text{Fig. } 3(c)) \) on both d21 and 42 in groups supplemented with 100 and 150 \( \mu g \) of folic acid. Compared with the saline group, all of the folic acid treatment groups showed a decrease \( (P<0.05; \text{Fig. } 4(a)) \) of H3K9me2 mark in the IL-4 promoter. In contrast, the enrichment of H3K9me2 in the IL-6 promoter exhibited a pronounced increase \( (P<0.05; \text{Fig. } 4(c)) \) with the IOF of folic acid on d21 and 42.

Micrococcal nuclease quantitative PCR for IL-2, IL-4 and IL-6 promoters in the spleen

Folic acid injection during incubation had no effect on the promoter methylation of IL-6 on d21 and 42 (Fig. 5(c)). As shown in Fig. 5(a) and 5(b), however, the tightness of the IL-2 and IL-4 promoter methylation was improved \( (P<0.05) \) in the 150-\( \mu g \) treatment group on d21 and 42.

Polynomial regression analysis

The results of polynomial regression analysis indicated that all of the testing indexes, except IL-2 and IL-4 mRNA expression on d1, \( \text{MTHFR} \) mRNA expression on d21 and 42, \( \text{MTRR} \) mRNA expression on d1 and 42, BW on d1 and RCR during d1–21, showed a linear change \( (P<0.05) \) with the increased amount of folic acid additive.
Correlation analysis between the expression levels of IL-2, IL-4 and IL-6 with that of in vivo immune response and epigenetic changes

The results of correlation analysis proved that the expression levels of IL-2 and IL-4 were positively correlated with in vivo immune response (the lysozyme activity and the concentration of IgG and IgM in plasma), as well as H3K4me2 on their promoters, whereas they were negatively correlated with H3K9me2 and chromatin conformation on their promoters (Table 6). In contrast, the expression of IL-6 was negatively correlated with in vivo immune response and H3K9me2 and chromatin conformation on its promoter, whereas it was positively correlated with H3K4me2 on its promoter (Table 6).

Discussion

In the present study, IOF of folate caused a significant increase in hepatic expression of MTHFR and MTRR in broilers. MTHFR participates in one-carbon metabolism by converting 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the primary circulating form of folate[23,25]. In addition, MTRR is essential in maintaining adequate intracellular folate pools. Simultaneously, MTRR participates in catalysing the re-methylation of homocysteine to methionine and is required for the production of SAM[10]. These two enzymes both serve as central roles in folate metabolism. Therefore, the incremental expression of MTHFR and MTRR observed in this study indicates that in ovo folate acid supplementation may speed up the circulation of folate in broilers.

Lysozyme, as a nonspecific immunity factor, has an important role in maintaining the physiological equilibrium of organisms through strengthening the phagocytosis of macrophages and leukocytes[26]. Similar to lysozyme, Ig is also vital for humoral immunity. The results presented herein demonstrate that, in plasma, at all of the concentrations of lysozyme IgG and IgM exhibited an increase in folic acid-exposed broilers, suggesting that in ovo folic acid supplementation improves the immunity of broilers. In addition, treatment with 1-2 mg of folate daily for 12 weeks was associated with higher levels of proteins (serum albumin, IgM and complement protein) involved in the activation and regulation of immune function in humans[27].

Cytokines, as immune regulatory proteins, have an important role in the immune system[28] as well. Among these proteins, IL-2, produced by T helper (Th1) cells, regulates cellular immune responses, and IL-4, produced by Th2 cells, participates in mediating the humoral immune response[29]. IL-6 was originally identified as a B lymphocyte differentiation factor that regulates the immune response, haematopoiesis and inflammation[30]. Dietary methyl-donor supplementation has been shown to diminish IL-6 levels in plasma while elevating IL-2 concentrations in mice[31]. In agreement with these previous findings, we found a predominant up-regulation of IL-2 and IL-4 and a pronounced down-regulation of IL-6 in the spleens of the folate-exposed groups. IL-2 and IL-4 have key roles in anti-inflammation, and IL-6 is a pro-inflammatory cytokine. The dynamic balance of anti-inflammation and pro-inflammatory cytokine levels is critical for the health maintenance within animals. Previous researches revealed an attenuated expression of IL-6 in lipopolysaccharide-treated mice[27,31]. In addition, the combination of melatonin and folate was reported to reduce the markers of liver injury by CCl4 and the levels of in...
Clinical phenotype and disease risk\textsuperscript{(35)}. Histone modifications are vital mechanisms involved in the transcriptional regulation of genes\textsuperscript{(36)}. For instance, H3K4me2 participates in euchromatin formation and ongoing gene expression\textsuperscript{(37)}. In the present experiment, higher H3K4me2 levels were found on the promoters of IL-2 and IL-4 in the spleens of broilers exposed to folate, and these levels coincided with the up-regulation of these two genes at the mRNA level; furthermore, the diminished IL-6 gene expression was in accordance with less enriched H3K4me2 on its promoter. Contrary to H3K4me2, H3K9me2 is a repressive histone mark that negatively regulates transcription by promoting a compact chromatin structure\textsuperscript{(37)}. The current study, indeed, revealed more enriched H3K9me2 on the IL-6 promoter. Concomitantly, the decreased H3K9me2 implies the possible involvement of the transcriptional regulation of IL-2 and IL-4 genes in the spleens of broilers in response to folate.
embryonic folate exposure. Therefore, it appears that IOF of folic acid regulates the expression of cytokines by adjusting the histone modification status on their promoters, thus promoting the immune function of broilers.

According to our understanding, the chromatin remodelling caused by the histone modification status of genes’ promoters may affect the binding of specific transcriptional factors, thereby regulating the transcriptional levels of these genes (38). Furthermore, the positive regulation of transcription is associated with a more loose promoter conformation with higher accessibility (39, 40), and vice versa. To our surprise, in the current study, we found an incongruity between gene expression and promoter conformation for IL-2, IL-4 and IL-6 on d21 and 42 all were <0.001. The P values gained by linear analysis of IL-2, IL-4 and IL-6 on day 21 and 42 all were <0.001. H3K9me2, histone H3 lysine 9 dimethylation.

Fig. 4. Effects of in ovo feeding of folic acid on the H3K9me2 enrichment ratio in IL-2, IL-4 and IL-6 promoters on d1 (A), 21 (B) and 42 (C) in spleens of broilers. Values are means (n = 6), with standard errors represented by vertical bars. Mean values with unlike superscript letters were significantly different (P < 0.05). The P values gained by linear analysis of IL-2, IL-4 and IL-6 on day 21 and 42 all were <0.001. H3K9me2, histone H3 lysine 9 dimethylation. ■, 0 mg; □, 50 mg; △, 100 mg; ▲, 150 mg.

Fig. 5. Effects of in ovo feeding (IOF) of folic acid on chromatin conformation in IL-2, IL-4 and IL-6 promoters on d1 (A), 21 (B) and 42 (C) in spleens of broilers. Values are means (n = 6), with standard errors represented by vertical bars. Mean values with unlike superscript letters were significantly different (P < 0.05). The P values gained by linear analysis of IL-2, IL-4 and IL-6 on d21 and 42 all were <0.001. ■, 0 mg; □, 50 mg; △, 100 mg; ▲, 150 mg.
Epigenetics is an advanced biological system that selectively uses genomic information and is involved in various fundamental phenomena, among which exist extensive functional interactions. Thus, a complex physiological process may be regulated partially or altogether by these mechanisms, even leading to an abnormal phenomenon caused by diverse action times and/or action sites, which is beyond our comprehension at first glance. For instance, choline is a methyl donor, but choline-deficient embryos exhibited global and by2 DMR2 DNA hypermethylation in a regulatory CpG within the Dmnt1 gene concomitant with the induction of Dmnt1 expression in rats, and the prenatal choline supply increased by2 mRNA levels rather than inhibiting gene expression through methyl supply.

Obviously, the current study has some limitations. First, folic acid has a vital role in the maintenance of normal patterns of DNA methylation, which is important for cellular homeostasis. The present study did not investigate DNA methylation on related immunogenes. Second, epigenetics is involved in various fundamental phenomena; therefore, further studies are necessary to reveal the internal cause of the incongruity between gene expression and promoter conformation caused by embryonic folate supplementation.

In addition, we also found that IOF of folic acid led to a significant improvement in BW, ADG and FCR at the late growing stage of broilers. Folate is a potential antioxidant with the capacity for oxyradical scavenging and is essential for immunity maintenance, and these attributes would help birds cope with more immunological and oxidative stress during the late period. Furthermore, folate has an important role in poultry viability and growth, and folate insufficiency may affect the survival and growth of poultry. Therefore, more folate is needed to promote high-intensity growth and overcome more stress in the late stage of broilers.

In the current study, folic acid-exposed eggs also demonstrated a higher hatchability. Researchers have shown that the hatchability and poultry weight are associated with folate content in the eggs, and higher supplemental folic acid levels may be required for rapid embryonic development. Although commercial poultry breeder diets contain supplemental folic acid, we do not consider folate as a limiting factor for embryonic development or hatchability, as this inference fails to consider the transport of folate to the egg. IOF can directly convey and deposit nutrients into eggs, avoiding a plateau value of folate levels within the eggs. In the present experiment, the concentration of folate in the liver positively responded to increasing levels of IOF folic acid (0–150 μg). Moreover, hepatic folate content on d42 showed a greater decrease than that on d1 and 21, indicating that folate was efficiently used in the late growing period of birds, and this could be associated with the marked improvement in performance only during the second stage of broilers.

The chicken (Gallus gallus) is an important animal model that bridges the mammals and vertebrates in evolution and has long been used as a model species for the study of embryology, immunology, behaviour and reproduction. Although mostly descriptive, the results presented herein provide the first evidence that in ovo folic acid injection causes various changes in the splenic expression of immune effector molecules in broilers, with the involvement of epigenetic modifications including histone methylation and chromatin conformation. To some extent, these findings may help in the understanding of the role of embryonic folate supplementation on embryology and immunology in humans.

In summary, in ovo folic acid injection can improve the hatchability and growth performance in the late growth stage of broilers. The increase of hepatic folate concentration, as well as the expression of MTHFR and MTRR, demonstrates the promotion of folate metabolism. IOF of folate also enhances immune function via increasing plasma lysozyme activity and IgG and IgM concentrations, increasing the splenic expression of IL-2 and IL-4, and down-regulating the expression of IL-6, and the differential expression of immune effector molecules can be regulated by the histone modification of H3K4me2 and H3K9me2 and chromatin conformation in their promoters. Moreover, within the ranges of this research, the 150-μg treatment group was the best considering comprehensively all of the testing indexes.

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The authors declare that no conflict of interest exists.

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