



Maternal high-zinc diet attenuates intestinal inflammation by reducing DNA methylation and elevating H3K9 acetylation in the *A20* promoter of offspring chicks[☆]

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Abstract

A20 is an anti-inflammatory protein that suppresses ubiquitin-dependent nuclear factor κ B (NF- κ B) signaling, which can be regulated by the microelement zinc (Zn). In mammals, Zn deficiency contributes to a decrease in *A20* abundance, which impairs the gut mucosa barrier. However, it is unclear whether the epigenetic reprogramming of the *A20* promoter is involved in enhanced Zn-induced intestinal immunity, especially in avian species. Herein, we show that maternal organic Zn exposure resulted in significantly improved intestinal morphological characteristics, increased mucin 2 (*MUC2*) abundance and secretory IgA (sIgA) production in progeny jejunums. Maternal and offspring Zn supplementation partially alleviated Zn-deficiency-induced inflammatory response, accompanied by repression of NF- κ B signaling. Additionally, we observed DNA hypomethylation and histone H3 at lysine 9 (H3K9) hyperacetylation at the *A20* promoter region and subsequent activated *A20* expression in Zn-supplemented hens compared with control. Notably, maternal dietary organic Zn exposure exhibited greater attenuation of gut impairment, along with increased *MUC2* expression and sIgA level, and decreased the abundance of *TNF- α* and *A20* relative to the inorganic-Zn group. Furthermore, enhanced acetylated H3K9 and *A20* transcription at day 14 was found in the offspring adequate dietary Zn group. Thus, *A20* may be a novel inflammatory-suppressed factor of chick gut that is persistently promoted by dietary Zn supplementation via epigenetic modifications at *A20* promoter.

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1. Introduction

In neonatal avian species, the adaptive immune system is functionally immature, and newly hatched birds are dependent on maternal antibodies transferred through the egg yolk [1,2]. In addition, the intestine, the largest immune system in the body, plays a major role in resistance to infection. However, the functional development of gut-associated lymphoid tissue in neonatal chickens is slow, and the functional maturation occurs in the second week of life [3–5]. Consequently, there is increased susceptibility to pathogenic microorganisms associated with gut lesions in early chickens [6–8]. However, early nutrition intervention has been demonstrated to be effective for enhancing innate immunity and intestinal development [9], which is involved in epigenetic reprogramming [10].

Our previous study showed that zinc (Zn) prevented *Salmonella enterica* serovar typhimurium-induced loss of intestinal mucosal barrier function related to increased expression of *occludin* and *claudin-1* in broiler chickens [11]. Additionally, maternal Zn deficiency during pregnancy could weaken the humoral and cell-mediated immune responses to the hepatitis B vaccine. Therefore, B cell counts, hepatitis B virus-specific immunoglobulin G production, T cell proliferation, CD4⁺/CD8⁺ T cell ratio and Th1-type immune responses in offspring mice were decreased [12]. These findings suggest that early-enhanced Zn nutrition may elevate immunity in subsequent life. Interestingly, Zn was mostly consumed from the yolk of broiler embryos until embryonic day 17 [13], which indicates that maternal enhanced Zn status may be crucial for intestinal development and immune function of offspring chickens. However, few attempts have been previously performed to confirm this effect in avian species. Particularly, excess dietary Zn intake in chicks has been shown to impair pancreatic exocrine function [14]. Supplemental organic Zn in hen diet exhibited increased cellular immune response and primary antibody titers to *Salmonella pullorum* antigen in progeny compared with inorganic Zn [15]. Additionally, we have demonstrated that organic Zn supplementation in breeder's diet promoted intestinal development of offspring broilers (not published). Thus, the addition of adequate or excess organic Zn in hen diets may be helpful for gut mucosal immunity of progeny birds.

Abbreviations: ZnMHA, methionine hydroxy analog chelated zinc; H3K9Ac, histone H3 acetylation at lysine-9 (K9); HDAC, histone deacetylase; HAT, histone acetyltransferase; DNMT, DNA methylation transferase; IL, interleukin; TNF, tumor necrosis factor; MUC, mucin; NF- κ B, nuclear factor κ B; PCNA, proliferating cell nuclear antigen; ChIP, chromatin immunoprecipitation.

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Microelement Zn has been shown to be critical for maintenance of gut mucosal immunity through modulating the cytokine response and leukocyte recruitment [16]. In rats, Zn deficiency resulted in increased intestinal inflammation via the impairment of TNF α production [17]. In human monocytes, Zn suppressed LPS-induced activation of nuclear factor κ B (NF- κ B) signals and subsequent elevation of tumor necrosis factor α (TNF α) [18]. In contrast, Zn is required for nuclear translocation of NF- κ B and cytokine production in mast cells [19]. Importantly, the excessive transcription of proinflammatory cytokines controlled by NF- κ B signals has been strongly linked to susceptibility to inflammatory diseases [20]. However, zinc finger protein A20 can negatively regulate the inflammatory response by deubiquitinating ubiquitin-dependent factors of NF- κ B signaling cascades [21,22]. Furthermore, Zn addition increased the protein level and activity of A20 in mammals *in vitro* and *in vivo* [23–25], indicating protective effects on inflammatory disease. Recent studies have suggested that A20 expression can be inhibited by microRNA (miR-125b) and facilitated by histone H3K4 methylation at the A20 promoter [26,27]. In addition, Zn deficiency resulted in impaired DNA methylation status correlated with inflammatory response and low-quality embryos [28–30] that were restored by dietary Zn or methyl donor supplementation. To date, there has been no report on the relationship between A20 and gut health in avian species. Furthermore, the potential epigenetic mechanism remains poorly defined.

The goal of the current study was to examine the role of adequate or excessive maternal Zn intake on the progeny's intestinal immunity and underlying epigenetic mechanisms in broiler chickens. Therefore, we hypothesized that progeny epigenetic alterations can be induced by maternal organic Zn supplementation and alter A20 abundance leading to increased gut mucosal barrier function. In addition, inflammatory cytokines and NF- κ B p65 expressions were determined. Furthermore, we examined the difference of progeny gut immunity between maternal organic and inorganic Zn exposure.

2. Materials and methods

2.1. Animals and diet

The animal protocol was carried out in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals of the Beijing Administration Committee of Laboratory Animals under the leadership of the Beijing Association for Science and Technology [Permit Number: SYXK (Beijing) 2007-0023].

Female broiler breeders (Ross 308, 45 weeks old; China Agricultural University Zhuozhou Broiler Breeder Farm, Hebei, China) were housed in a temperature- and humidity-controlled environment and were fed a Zn-deficient (20 mg/kg) diet for 2 weeks to exhaust maternal Zn and keep the consistency of the Zn status. Subsequently, these broiler breeders were fed a controlled diet containing 20 mg/kg of Zn (Zn-deficient, ZD, $n=240$), a Zn-adequate (ZA) diet containing 70 mg/kg of Zn [ZnSO₄, IZA, $n=240$ or methionine hydroxy analog chelated Zn (ZnMHA), OZA, $n=240$] or a Zn-supplemented (ZS) diet containing 320 mg/kg of Zn (ZnSO₄, IZS, $n=240$ or ZnMHA, OZS, $n=240$) for 6 weeks. Organic ZnMHA and inorganic Zn (Zn sulfate) were obtained from Novus International (Saint Charles, MO, USA) and Blooming Bio-tech (Beijing, China), respectively. Hens were artificially inseminated, and hatching eggs (400 eggs per group) from 51-week-old hens were incubated under standard conditions of 70%–80% humidity and 37.8°C with intermittent rotations. After hatching, offspring birds supplemented by ZnSO₄ diets were randomly divided into two groups to receive either a low-Zn diet (containing 40 mg/kg Zn) or normal-Zn diet (containing 90 mg/kg Zn) for 6 weeks, consisting of 7 replicates with 10 birds per replicate. The chickens were fed *ad libitum* and housed in wire cages under standard gradually decreased temperature regimens from 35°C to 22°C. The birds were fed corn-soybean-meal-based diets, which were formulated based on NRC (1994) requirements and isoenergetic, isonitrogenous and isomethionine. The actual concentration of Zn in ZD, OZA, IZA, OZS and IZS was 33.52, 83.62, 82.97, 322.70 and 324.68 mg/kg, respectively. A two-phase feeding program in offspring birds was performed, with a starter diet from day 1 to 21 and a grower diet from day 22 to 42. Analyzed values of dietary Zn concentration in offspring diets between deficient- and adequate-Zn groups were 56.79, 100.49 (day 1–21) and 46.61, 93.06 (day 22–42) mg/kg, respectively.

2.2. Intestinal sample collections

On days 14 and 35, seven birds from each treatment were randomly selected and sacrificed using pentobarbital anesthesia. Two-centimeter intestinal segments of the distal jejunum (3 cm proximal to Meckel's diverticulum) were removed and fixed in

4% (w/v) paraformaldehyde for analysis of intestinal morphology and goblet cell counting. The closed 2-cm segments were removed, immediately frozen in liquid nitrogen and stored at -80°C until analysis for DNA, RNA and protein. Fifteen-centimeter jejunal segments were opened and flushed, and mucosa samples were collected and stored at -40°C until analysis for secretory IgA (sIgA).

2.3. Intestinal morphology analysis and goblet cell counts

The fixed jejunums were dehydrated using a graded series of ethanol and cleared with xylene, and the samples were then embedded in paraffin. Next, 5- μm slices were cut and stained with hematoxylin and eosin. A vertical section was prepared from the jejunum to determine the morphological characteristics of the intestine. Morphological pictures were performed under a light microscope. A total of 48 intact villi and crypts per treatment were selected for villous height, villous width at one-half villous height, villous bottom width and crypt depth. Jejunal tissue sections (5 μm) were stained for total goblet cells or mucin with periodic-acid–Schiff reagent (PAS). The number of PAS-positive cells along the villi was counted by light microscopy. The density of goblet cells was calculated as the number of goblet cells per unit of villous height. All measurements were performed with a Leica light microscope (Leica LB30T, Wetzlar, Germany) using Image Pro Plus 5.1 (IPP5.1) software.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from intestinal samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. cDNA synthesis was performed using PrimeScript RT reagent kit with cDNA eraser (TaKaRa, Dalian, Liaoning, China) according to the manufacturer's instructions. The one-step real-time RT-PCR was performed using SYBR Premix Ex TaqTM (TaKaRa, Dalian, Liaoning, China) in a real-time PCR machine (ABI 7500; Applied Biosystem, Carlsbad, CA, USA) following the manufacturer's guidelines. The primer pairs used are shown in Supplementary Table 1. β -Actin was utilized as the housekeeping gene. Relative mRNA expression levels of each target gene were normalized to the control using the $2^{-\Delta\Delta\text{CT}}$ method [8,31].

2.5. Protein isolation and Western blotting

DNMT3b and caspase 3 antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA), and DNMT3a, HAT1, HDAC2 and β -actin antibodies were obtained from Abcam (Cambridge, MA, USA). Histone H3 lysine 9 acetyl (H3K9Ac) antibody was obtained from Millipore (Billerica, MA, USA), and proliferating cell nuclear antigen (PCNA) antibody was obtained from Sigma (St. Louis, MO, USA).

Fifty milligrams of frozen jejunal segments for protein extraction was homogenized in RIPA buffer and centrifuged at $15,000\times g$ for 20 min at 4°C . Supernatants were collected, and the total protein concentrations were normalized using the BCA Assay (Appllygen Technologies, Beijing, China). Histones were extracted using a Total Histone Extraction Kit (Epigentek, Farmingdale, NY, USA). Tissue lysates (40 μg) were diluted in Laemmli sample buffer before sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then were separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes. Incubation in TBST [30 mM Tris base (pH 7.6), 200 mM NaCl and 0.1% Tween 20] containing 5% milk was performed for 1 h at room temperature to block the membrane. Next, the membrane was incubated with antibodies (1:200) in TBST containing 5% milk at 4°C overnight. The next day, the membrane was washed three times for 10 min with TBST on a shaker, incubated with secondary antibody diluted TBST (1:2000) containing 5% milk at room temperature for 1 h and washed three times for 10 min in TBST. Signals were detected with a Super Enhanced Chemiluminescence Kit (Appllygen Technologies, Beijing, China) and scanned using a Gel Documentation System (Bio-Rad, Hercules, CA, USA). Quantification of band density was determined using Quantity One software (Bio-Rad, Hercules, CA, USA).

2.6. Global methylation status

Genomic DNA from jejunums was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's recommended protocol, and the DNA concentration was detected. DNA with an A260/280 nm ratio ranging from 1.8 to 2.0 was considered purified DNA and used for the DNA methylation assay. Global DNA methylation level analysis was performed using a MethylFlash Methylated DNA Quantification Kit (Epigentek, Farmingdale, NY, USA) according to the manufacturer's instructions. The amount and percentage of methylated DNA [5-methylcytosine (5-mC)] in the total DNA extract were calculated based on a standard curve versus the amount of positive control (methylated polynucleotide containing 50% of 5-mC) at each concentration point.

2.7. Bisulfite sequencing PCR

Promoters are located in the 1333-base-pair (bp) and 2533-bp regions of the A20 gene. The A20 promoter exhibits high CpG density, which meets the standard CpG island size of >200 bp, a GC percentage of >50.0 , and an obs/exp ratio of >0.6 , and is within a 446-bp window between +1468 bp to +1913 bp. Extracted genomic DNA (1 μg) was bisulfite modified using the EZ DNA Methylation-Gold Kit (Zymo Research, Los Angeles, CA, USA) according to the supplier's instructions. The bisulfite modified

DNA was suspended in 20 μ l of deionized water and used immediately or stored at -80°C until use. The converted DNA was amplified by PCR. The primer sequences are as follows: 5'-AGGGTTGGATGAGAGTTGT-3' (forward) and 5'-AACCCCAACA(G)AAAATTTCCAC-3' (reverse). The PCR conditions for the first PCR are as follows: 95°C for 5 min; 94°C for 30 s, 60°C for 45 s and 72°C for 45 s $\times 10$. The PCR conditions for the second PCR are as follows: 30 s at 95°C , 45 s at 50°C , 45 s at 72°C $\times 30$; 4 min at 72°C . The PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and subsequently subcloned into the pGEMT-easy vector (Promega, Shanghai, China). Ten individual clones for each sample were selected for sequencing (Microread Genetics, Beijing, China).

2.8. Chromatin immunoprecipitation (ChIP)–PCR assay

ChIP analysis was performed by Kangcheng Biotechnology (Shanghai, China). Briefly, jejunal samples were incubated with 1% formaldehyde for 30 min at 37°C . Next, the lysates were sonicated in SDS lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS, 0.5 mM PMSF) for 10 min (30 s “ON” and 30 s “OFF”) to yield DNA fragments with a size ranging from 200 to 500 bp by gel analysis. Aliquots of chromatin were incubated with 1 μ g of anti-acetyl histone H3K9 (Millipore, Billerica, MA, USA). Anti-RNA polymerase (Millipore, Billerica, MA, USA) was used as a positive control, and IgG (Santa Cruz, Dallas, TX, USA) was used as a negative control. Five percent of immunoprecipitated DNA was used for real-time PCR using a ViiA 7 Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA), and each sample was assayed in triplicate. The primer sequences for the promoter region of *A20* are given in Table S1. The qPCR values were normalized to the values of the *GAPDH* promoter region. PCR conditions included an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 10 min. The CT values of the ChIP signals detected by real-time PCR were converted to the percentage of the input DNA as ChIP signal, which was calculated by the delta-delta method [31], using the formula $[2^{-(\text{CT}_{\text{IP sample}} - \text{CT}_{\text{input}})}] \times 100\%$.

2.9. Statistical analysis

The results are expressed as the mean \pm standard deviation (S.D.). All data were analyzed using SPSS statistical software (SPSS for Windows, version 17.0; Chicago, IL, USA). All data were subjected to GLM in a 5×2 factorial arrangement with maternal and offspring Zn exposure. When the interactions were significant, means were separated using one-way analysis of variance (ANOVA) and a *post hoc* Duncan's multiple range test. When interactions were not significant, the polynomial contrasts were performed over the main effect means for maternal Zn exposure (averaged over offspring treatments). $P < .05$ was used as the threshold for statistical significance.

3. Results

3.1. Morphological parameters

Data for jejunal morphology of offspring birds are shown in Supplementary Fig. 1. There were significant interactions between maternal and offspring Zn supplementation on the villus height to crypt depth ratio ($P = .010$) on day 14, villus height ($P = .044$), villus width ($P < .001$), crypt depth ($P < .001$) and the villus height to crypt depth ratio ($P < .001$) on day 35. Offspring birds from OZS or IZS breeders exhibited higher villus height to crypt depth ratio on days 14 and 35 compared with Zn-deficient breeders ($P = .010$ and $P < .001$, respectively). Maternal high dose of ZnSO₄ or ZnMHA supplementation showed increased villus height ($P < .001$, Supplementary Fig. 1A) and villus width ($P < .001$, Supplementary Fig. 1C) and decreased crypt depth ($P < .001$, Supplementary Fig. 1E) on day 14 compared with Zn-deficient hens. Relative to progeny chicks exposed to a Zn-deficient environment, the dietary supplementation of 70 mg/kg of Zn significantly elevated villus height ($P < .001$, Supplementary Fig. 1A) and decreased crypt depth ($P < .001$, Supplementary Fig. 1E) on day 14. Furthermore, progeny chickens from OZS broiler breeders demonstrated a greater improvement of jejunal morphology than chickens from IZS. However, there were no differences in villus width on day 14 ($P = .371$, Supplementary Fig. 1C) in offspring chicks supplemented with either 20 or 70 mg/kg of Zn. Therefore, these results indicate that Zn, especially organic Zn, addition in breeder and offspring diets resulted in lasting improved jejunal development.

3.2. Goblet cell density, sIgA levels and MUC2 levels

To further assess the effects of the microelement Zn on the gut mucosal function, we examined goblet cell counts and density in the progeny jejunums. Our results showed that there were marked interactions between maternal and offspring Zn exposure on goblet cell counts ($P < .001$, Supplementary Fig. 2A) and density ($P = .001$, Supplementary Fig. 2C) on day 14 in the offspring jejunum. However, this interaction did not exist on day 35 ($P = .109$ and $P = .154$, respectively; Supplementary Fig. 2B and D). Offspring from ZD hens had the lowest goblet cell counts ($P = .022$, Supplementary Fig. 2A) but highest density ($P < .001$, Supplementary Fig. 2C) on day 14. However, the counts and density of jejunal goblet cells on day 35 in both maternal and offspring Zn-deficient groups were reduced in Zn-supplemented birds ($P < .05$, Supplementary Fig. 2B and D). In addition, supplemental organic Zn in hens showed beneficial effects on goblet cell counts and density compared with the inorganic Zn group.

The sIgA content of jejunal mucosa on day 14 was markedly affected by the interaction of maternal and offspring Zn treatments ($P = .021$, Fig. 1A). Offspring birds from ZD hens fed with 20 mg/kg Zn exhibited the lowest sIgA content in the jejunum on day 14 (Fig. 1A). Dietary supplemental 300 mg/kg ZnMHA in the breeder diets significantly increased sIgA levels on day 35 compared with other groups ($P < .05$, Fig. 1B). The data for mRNA expression of *MUC2* are presented in Fig. 1. A significant increase of *MUC2* mRNA abundance in the jejunum on day 14 was observed in the offspring chicks exposed to 70 mg/kg of Zn from OZS hens ($P < .05$, Fig. 1C). The result on day 35 was consistent with that on day 14 (Fig. 1D). Therefore, these observations suggest that the enteric mucosal barrier function might be enhanced after maternal and offspring Zn supplementation.

3.3. PCNA and caspase 3 expression

To determine whether zinc addition in hens diet had any effects on cell proliferation or apoptosis, protein levels were detected by immunoblotting against PCNA and Caspase 3 antibodies. Western blotting showed no interactions between maternal and offspring Zn exposure by examining the protein levels of PCNA and caspase 3 ($P > .05$, Fig. 2). Maternal Zn deficiency significantly decreased the protein expression of PCNA in the offspring jejunum on day 14 (Fig. 2B) and day 35 (Fig. 2E). Chicks from the OZS group demonstrated higher PCNA on day 14 relative to chickens from the IZS group (Fig. 2B). Moreover, offspring birds from ZD hens exposed to the Zn-deficient diet showed markedly elevated caspase 3 expression on day 14 (Fig. 2C). Offspring chicks that received 20 mg/kg of Zn exhibited increased levels of caspase 3 on day 35 (Fig. 2F) compared with the chicks that were fed 70 mg/kg of Zn. Therefore, these data indicate that Zn addition in hen diets enhanced cell proliferation, which was paralleled with the attenuated apoptosis in the progeny gut.

3.4. Inflammatory markers

The abundance of *IL6*, *IL8*, *IL-1 β* and *TNF α* in progeny birds was examined by RT-PCR (Figs. 3 and 4). Exposure to 70 mg/kg of Zn decreased mRNA levels of *IL-1 β* , *TNF α* and *IL8* on day 14 (Fig. 3A, B and D) as well as *IL-1 β* and *IL6* on day 35 (Fig. 3A and C) in jejunums compared with offspring chickens fed with 20 mg/kg of Zn ($P < .05$). *IL8*, *IL-1 β* , and *TNF α* mRNA abundance was persistently affected by maternal Zn exposure ($P < .05$). *IL8*, *IL-1 β* and *TNF α* (day 14; Fig. 3) and *IL6*, *IL-1 β* , and *TNF α* (day 35; Fig. 4) expression in progeny jejunums from ZD was observably up-regulated compared with offspring birds from IZS and OZS. Although *TNF α* (day 14) and *IL-1 β* (day 35) expression in the OZS group was significantly down-regulated when compared with IZS

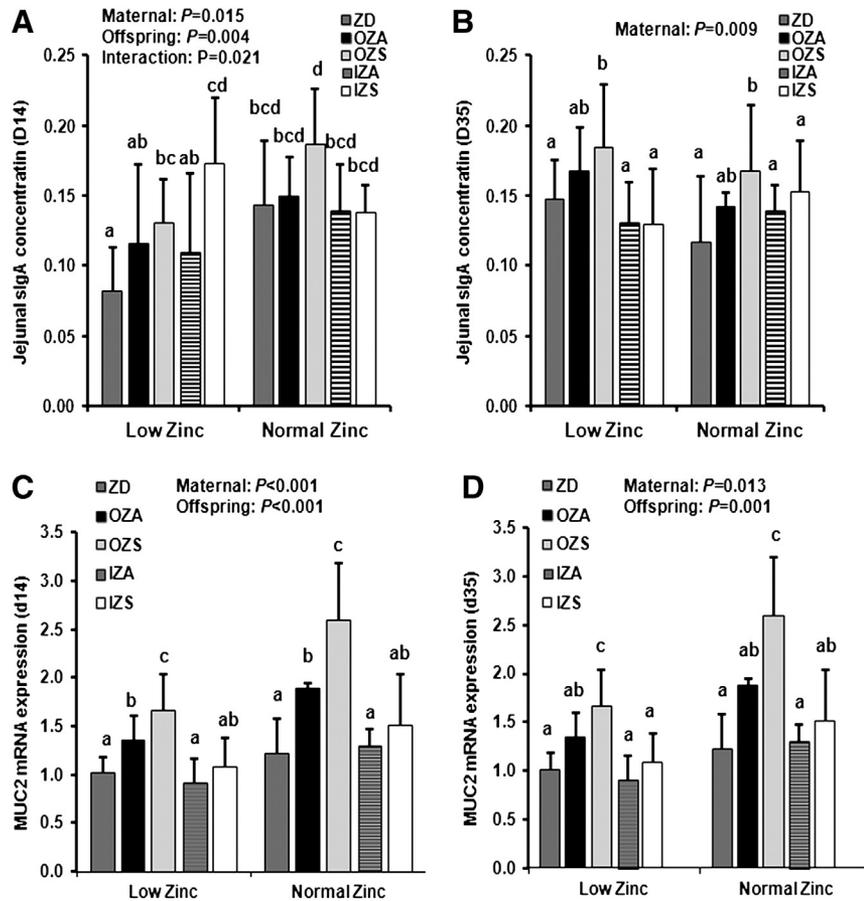


Fig. 1. Zn supplementation in broiler breeder and progeny bird diets enhanced *MUC2* transcription and sIgA production. (A and B) sIgA levels of jejunal mucosa at days 14 and 35 ($n=7$). OZS birds consistently exhibited markedly higher *MUC2* mRNA expression at day 14 (C; $n=6$) and day 35 (D; $n=6$). ZD, OZA and OZS represent breeders supplied with extra 0, 50 or 300 mg/kg organic Zn in basal diet, respectively. IZA and IZS represent the breeders supplied with extra 50 or 300 mg/kg inorganic Zn in basal diet, respectively. Low Zn and normal Zn represent the offspring chicks fed with extra 20 or 70 mg/kg Zn in basal diet, respectively. Statistical analysis was performed by SPSS GLM, and a one-way ANOVA was performed using the Duncan correction for multiple comparisons when the interaction was significant ($P<0.05$). Values are means, with standard deviations represented by vertical bars. ^{a,b}Treatments with unlike letters were significantly different ($P<0.05$).

group, no statistical difference of *IL6* and *IL8* gene expression was observed. Therefore, we conclude that maternal and offspring Zn supplementation alleviated Zn-deficiency induced intestinal inflammation.

3.5. NF- κ B p65 and A20 expression

To clarify the potential mechanism, NF- κ B p65, a key factor of the NF- κ B signaling pathway regulating the production of proinflammatory cytokines, was measured. NF- κ B p65 mRNA abundance in the progeny jejunums was markedly down-regulated by maternal and offspring Zn addition on day 14 (Fig. 5A and C) but only influenced by maternal Zn supplementation on day 35 day relative to maternal and offspring Zn deficiency ($P<0.05$). Breeders fed with 300 mg/kg of ZnMHA exhibited decreased NF- κ B p65 mRNA expression in the offspring jejunums on days 14 and 35 ($P<0.05$). These results demonstrate that Zn supplementation might inhibit the activation of the NF- κ B pathway. A20 is a well-characterized negative regulator of the NF- κ B pathway. Thus, we sought to verify whether A20 had been altered by RT-PCR. As shown in Fig. 5B and D, dietary Zn supplementation in both hen and offspring diets enhanced the expression of A20 in offspring jejunums on days 14 and 35 ($P<0.05$). The addition of 300 mg/kg of ZnMHA in the breeders' diet showed higher A20 transcript compared with other groups ($P<0.05$). Consequently, the trace element Zn is likely to exert anti-inflammatory

effects in the chicken intestines via facilitating A20 transcription and inactivating NF- κ B signaling.

3.6. Global methylation ratio and A20 methylation status

To determine whether DNA methylation might be altered by Zn deficiency or supplementation, we used an enzyme-linked immunosorbent assay kit, Western blotting and bisulfite sequencing for analysis. *De novo* methyltransferases (DNMT3a and DNMT3b) were measured by immunoblotting (Fig. 6A and B). In 14-day-old chicks, fold changes of DNMT3a were not altered by maternal Zn treatments but were significantly decreased by offspring Zn supplementation (Fig. 6A). In contrast, there was significant down-regulation of DNMT3b in the progeny jejunums from hens supplemented with 300 mg/kg of organic Zn. Additionally, offspring chicks fed with Zn-adequate diet from OZS hens could suppress DNMT3a expression on day 35. Moreover, DNMT3b protein levels were decreased by a maternal high dose of ZnMHA regardless of progeny Zn status ($P<0.05$, Fig. 6B). However, maternal Zn deficiency resulted in lasting high global genomic methylation, which could be reversed by maternal IZS and OZS treatments on days 14 (Fig. 6C) and 35 (Fig. 6D). Zn deficiency in the offspring birds significantly elevated global genomic methylation status on day 14 (Fig. 6C) but not day 35 (Fig. 6D).

Because the alterations of global DNA methylation status were not completely consistent with the abundance of A20 mRNA, we next

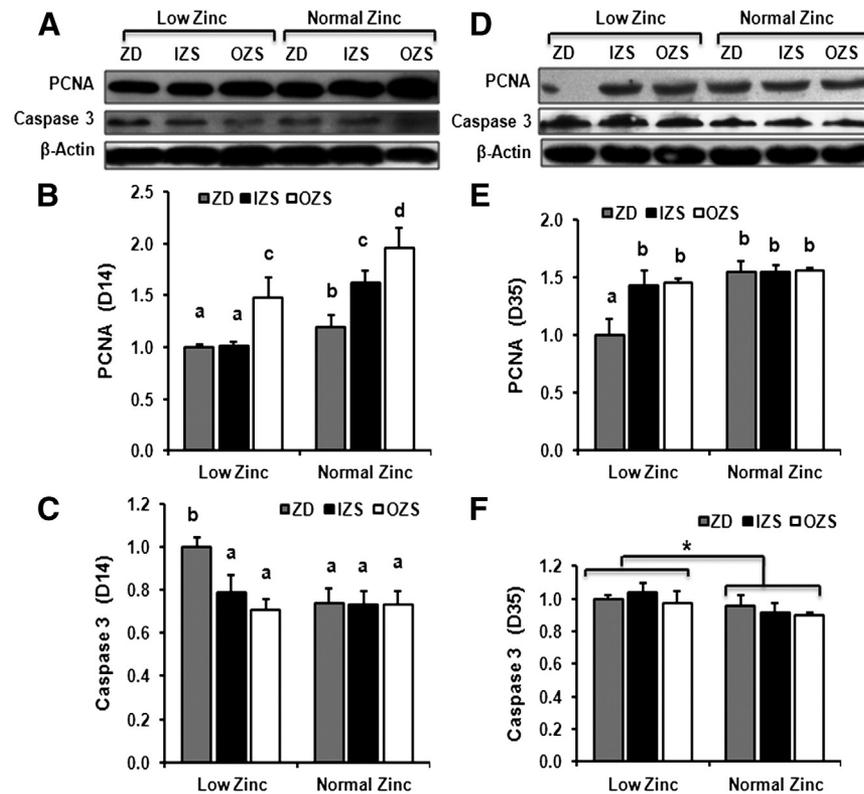


Fig. 2. Supplemental high dose of organic Zn in hens improved the expression of PCNA and caspase 3 in progeny jejunums. Representative Western blots of PCNA and caspase 3 protein in progeny jejunal tissues at days 14 (A; $n=4$) and 35 (D; $n=4$) are shown. β -Actin was utilized as the protein loading control. Maternal Zn deficiency resulted in significant up-regulation of caspase 3 at day 14 (C) and down-regulation of PCNA on days 14 (B) and 35 (E). (F) No changes were noted in caspase 3 at day 35 after maternal exposure to Zn. Statistical analysis was performed by SPSS GLM, and a one-way ANOVA was performed using the Duncan correction for multiple comparisons when the interaction was significant ($P<.05$). Values are means, with standard deviations represented by vertical bars. ^{a,b}Treatments with unlike letters were significantly different ($P<.05$).

examined the DNA methylation frequency by bisulfite sequencing with a special reference to the methyl-CpG status in the *A20* promoter region (Fig. 6). Methylation site mapping of CpG islands resulted in methylation analysis of 41 CpGs in the jejunums of four animals per group (Supplementary Fig. 3 and 4), which showed site-specific modifications. The DNA methylation levels in Zn-deficient or -adequate progeny birds from maternal Zn deficiency on day 14 and day 35 were 15.58% (or 13.18%) and 14.53% (or 13.33%), respectively. Maternal Zn addition significantly reduced DNA methylation levels of *A20* ($P<.05$, Fig. 7). Offspring chicks from OZS showed hypomethylation of *A20* on day 14 compared with that from IZS ($P<.05$, Fig. 7B). However, this effect failed to persist to day 35 ($P>.05$, Fig. 7C). Taken together, these findings provide evidence that maternal Zn supplementation restricted DNA methylation in the *A20* promoter region paralleled with decreased transcription of *A20* in the offspring intestine.

3.7. Global levels and *A20* promoter H3K9 acetylation

Histone acetylation plays an important role in the regulation of gene expression, which is in part regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). H3K9Ac participates in the activation of gene expression. We next investigated the state of H3K9 acetylation by Western blotting with antibodies specific to HAT1, HDAC2 and H3K9Ac to determine the relative degree of acetylation after normalization to β -actin and total histone H3 levels (Fig. 8). As shown in Fig. 8A and B, offspring birds from a maternal high-inorganic-Zn diet resulted in significant HDAC2 up-regulation in progeny jejunums on days 14 and 35 when the offspring birds were fed with low-Zn diet ($P<.05$). Contrarily, exposure to a maternal high-organic-Zn diet suppressed the expression of HDAC2 on day 14 ($P<.05$,

Fig. 8A). Maternal Zn supplementation significantly elevated HAT1 protein levels in the progeny jejunums on both days 14 and 35 (Fig. 8A and B). However, no significant deviation was observed from low- and normal-Zn-treated progeny. Western blot analysis showed that the levels of H3K9Ac in maternal Zn-deficient hens were low. However, the maternal OZS treatment led to a 2.49-fold (day 14, Fig. 8C) and 1.53-fold (day 35, Fig. 8D) increase of H3K9Ac. Moreover, H3K9 was highly acetylated in offspring birds supplemented with normal-Zn diets compared with low-Zn-treated offspring on day 35 (Fig. 8B and D). To determine whether acetylation of H3K9 could contribute to *A20* transcription, a combination of ChIP and qRT-PCR was conducted, and three pairs of primers in the *A20* promoter region were designed. Consistent with the transcript levels of *A20*, the *A20* promoter was nearly absent in the maternal Zn-deficient group but was highly enriched for H3K9Ac in the hens exposed to OZS (Fig. 9). Of interest, H3K9Ac levels were site specific. Although H3K9Ac levels in the *A20* promoter region (1691–1745 bp) on day 35 were not significantly altered (Fig. 9F), H3K9Ac levels in the *A20* promoter region (340–425 and 506–603 bp) on day 14 increased after maternal and offspring Zn addition compared with Zn-deficient treatment (Fig. 9A and C). In addition, there were enhanced accumulations of H3K9Ac after maternal exposure to OZS, but not IZS, on days 14 and 35 (Fig. 9B, D, and E) compared with the ZD group. Collectively, these results suggest that *A20* gene transcription was down-regulated by H3K9 deacetylation in the Zn-deficient avians.

4. Discussion

The role of Zn used for the enhancement of intestinal development and function in chickens is well established [32–34]. Our previous study showed that the mucosal barrier function in weaning piglets

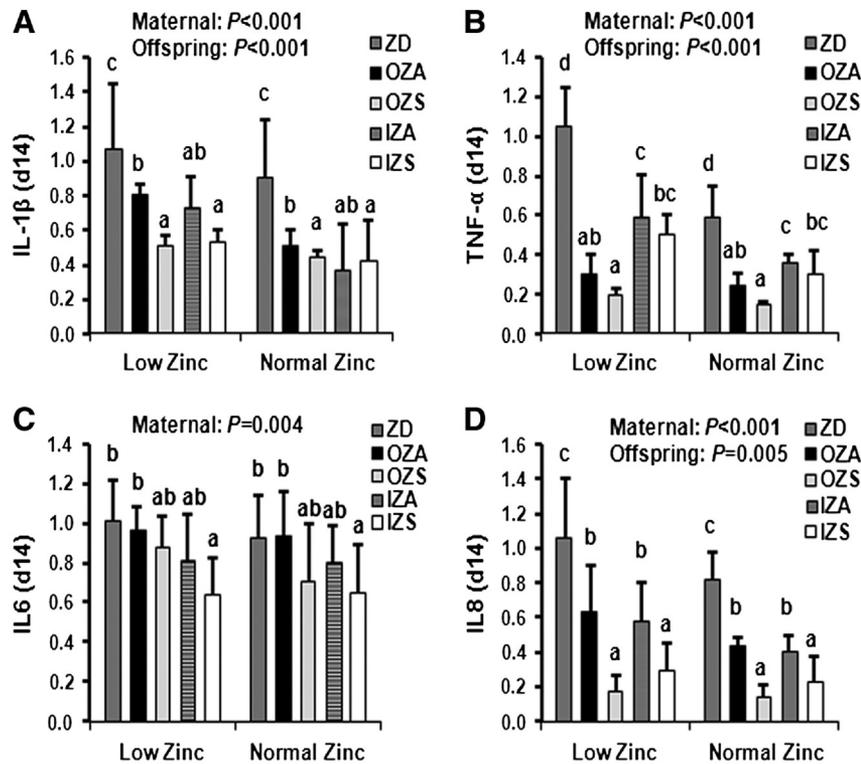


Fig. 3. Maternal excessive organic Zn intake reduced the proinflammatory response of offspring jejunums at day 14. The expression of proinflammatory cytokines *IL-1 β* (A), *TNF α* (B), *IL6* (C) and *IL8* (D) was significantly down-regulated by maternal and offspring Zn addition. Statistical analysis was performed by SPSS GLM, and a one-way ANOVA was performed using the Duncan correction for multiple comparisons when the interaction was significant ($P < .05$). Values are means, with standard deviations represented by vertical bars from six chicks. ^{a,b}Treatments with unlike letters were significantly different ($P < .05$).

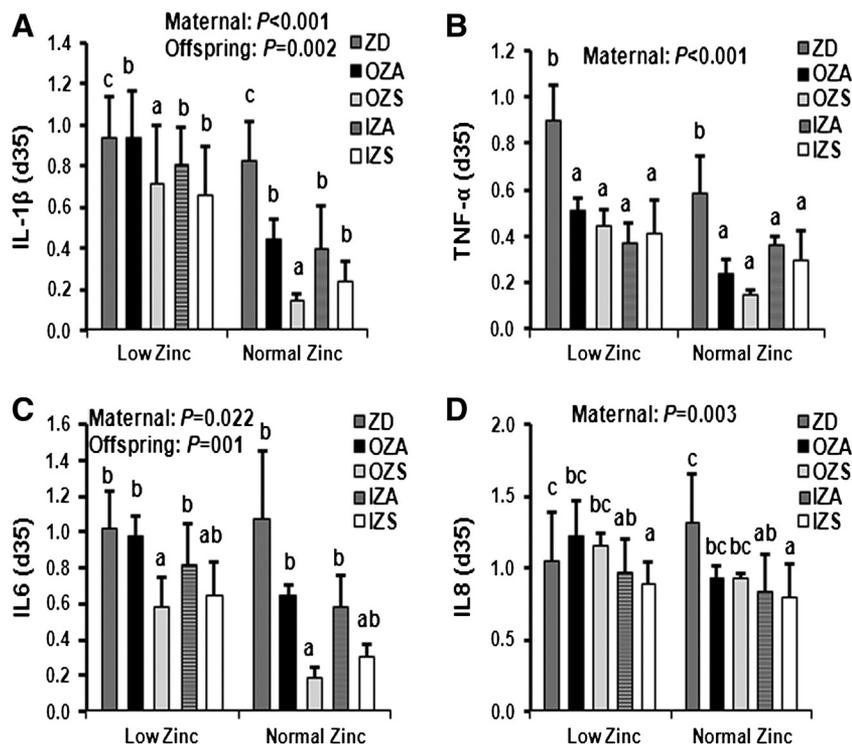


Fig. 4. Zn addition alleviated proinflammatory cytokine expression of offspring jejunums at day 35. mRNA expression of proinflammatory cytokines *IL-1 β* (A), *TNF α* (B), *IL6* (C) and *IL8* (D) was determined by quantitative PCR and significantly up-regulated in the ZD group compared with OZS and IZS groups. Statistical analysis was performed by SPSS GLM, and a one-way ANOVA was performed using the Duncan correction for multiple comparisons when the interaction was significant ($P < .05$). Values are means, with standard deviations represented by vertical bars from six chicks. ^{a,b}Treatments with unlike letters were significantly different ($P < .05$).

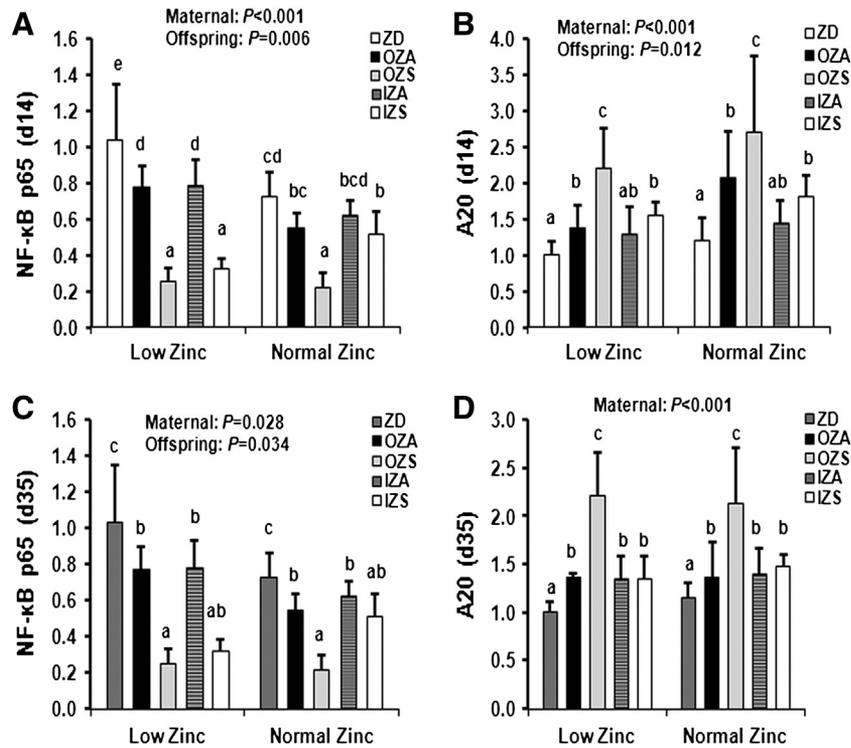


Fig. 5. Zn supplementation reversed the abnormal expression of *NF-κB p65* and *A20*. (A) The mRNA expression at day 14 (n=6). (B) The mRNA expression at day 35 (n=6). Statistical analysis was performed by SPSS GLM, and a one-way ANOVA was performed using the Duncan correction for multiple comparisons when the interaction was significant (P<.05). Values are means, with standard deviations represented by vertical bars. ^{a,b}Treatments with unlike letters were significantly different (P<.05).

was enhanced after Zn supplementation in the basal diet, accompanied by an increase in occludin and zonula occludens protein-1 expression [35]. Recently, similar effects were observed in broiler

chickens [11]. These results indicate that Zn is effective for gut mucosal function. However, whether maternal Zn treatment in the broiler breeder diet can promote its offspring's intestinal function is

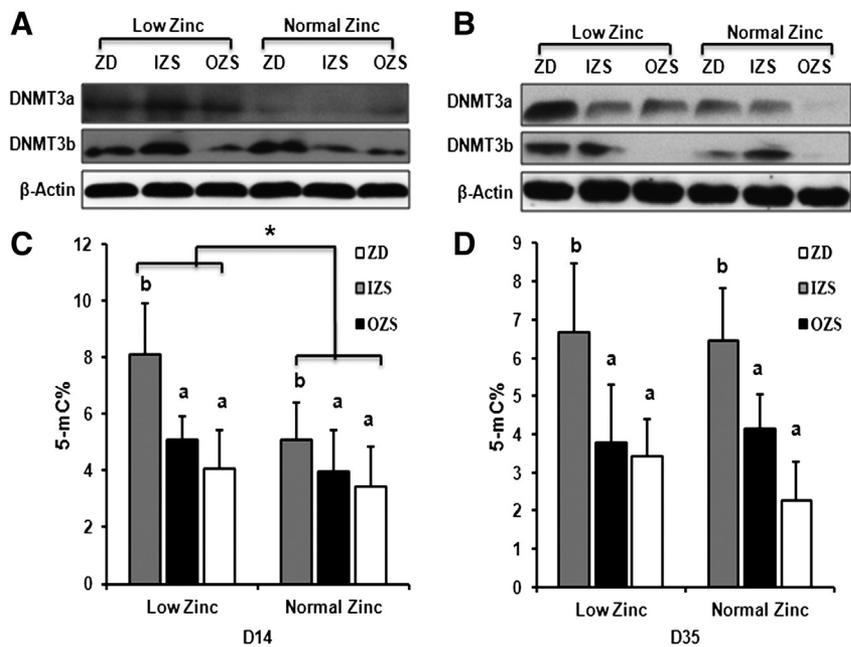


Fig. 6. Zn supplementation in hen diets decreased genomic methylation levels in progeny jejunum. (A and B) Western blots of DNA methyltransferases (DNMT3a and DNMT3b) normalized by corresponding β-actin expression in jejunal tissues at day 14 and day 35 (n=4). (C and D) Global genomic methylation levels detected using the MethylFlash Methylated DNA Quantification Kit in the jejunum at days 14 and 35 (n=6). Global DNA methylation level was calculated as a percentage of 5-mC to total DNA. Statistical analysis was performed by SPSS GLM, and a one-way ANOVA was performed using the Duncan correction for multiple comparisons when the interaction was significant (P<.05). Values are means, with standard deviations represented by vertical bars. ^{a,b}Treatments with unlike letters were significantly different (P<.05).

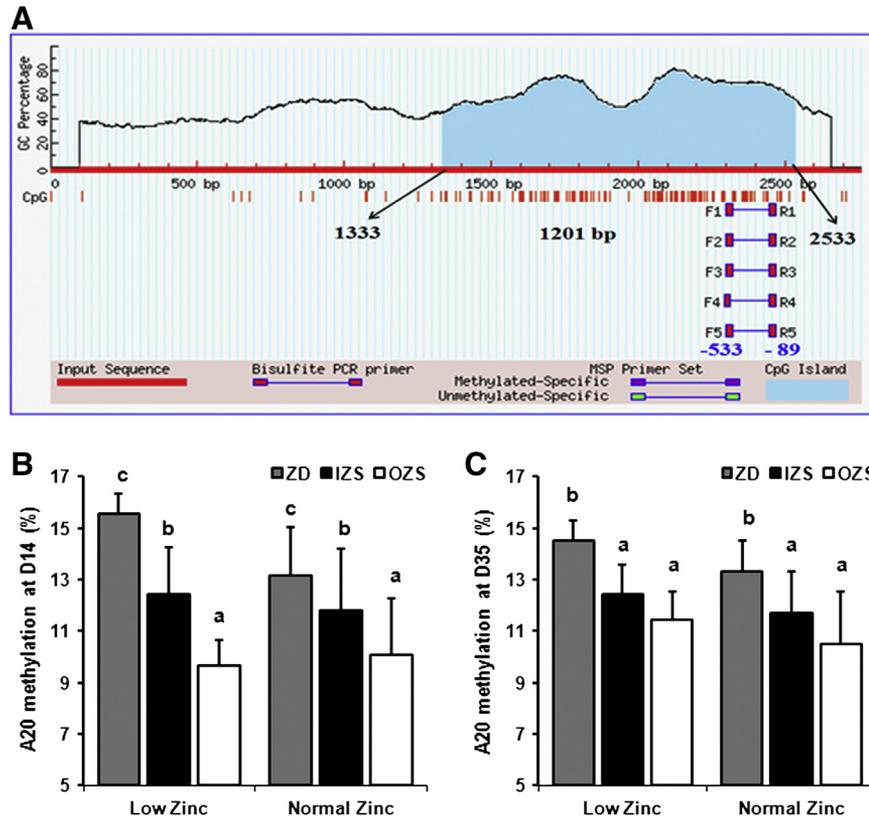


Fig. 7. Maternal Zn exposure repressed methylation status of the *A20* gene promoter in offspring jejunum. (A) Schematic representation of *A20* and its CpG-pattern rich regions is presented. Red horizontal line indicates the input sequence. Red vertical lines show the positions of CpG Sites within the 1201-bp fragment. (B and C) Methylation frequency at the *A20* promoter at day 14 and 35 ($n=4$). Statistical analysis was performed by SPSS GLM, and a one-way ANOVA was performed using the Duncan correction for multiple comparisons when the interaction was significant ($P<.05$). Values are means, with standard deviations represented by vertical bars. ^{a,b}Treatments with unlike letters were significantly different ($P<.05$).

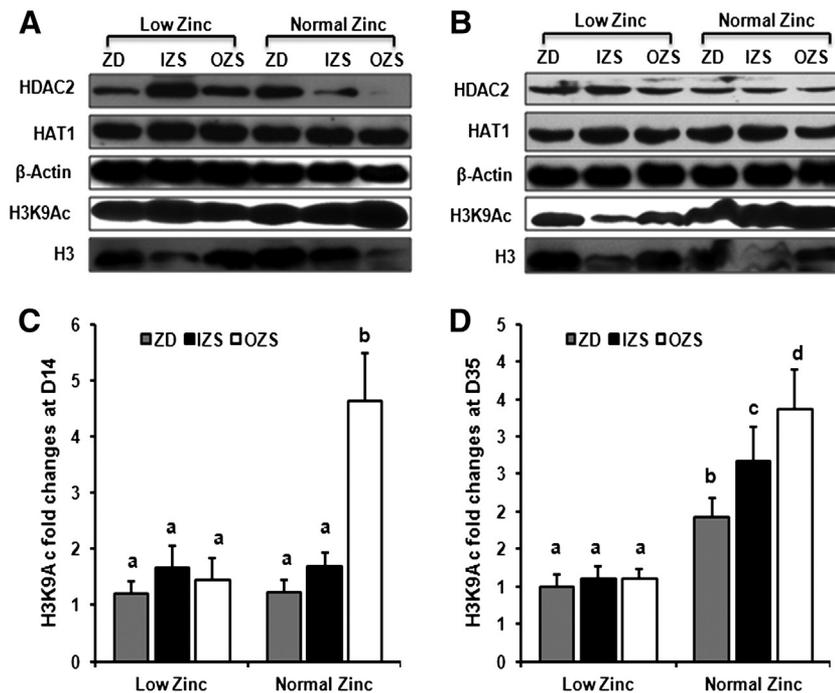


Fig. 8. Zn supplementation altered the protein abundances of HAT1, HDAC2 and H3K9Ac. (A and B) Protein blot analysis for HDAC2, HAT1 and H3K9Ac at days 14 and 35 ($n=4$). (C and D) Quantification of days 14 and 35 jejunal H3K9Ac levels. β -Actin and H3 were used as internal controls for HDAC2, HAT1 and H3K9Ac, respectively. Statistical analysis was performed by SPSS GLM, and a one-way ANOVA was performed using the Duncan correction for multiple comparisons when the interaction was significant ($P<.05$). Values are means, with standard deviations represented by vertical bars. ^{a,b}Treatments with unlike letters were significantly different ($P<.05$).

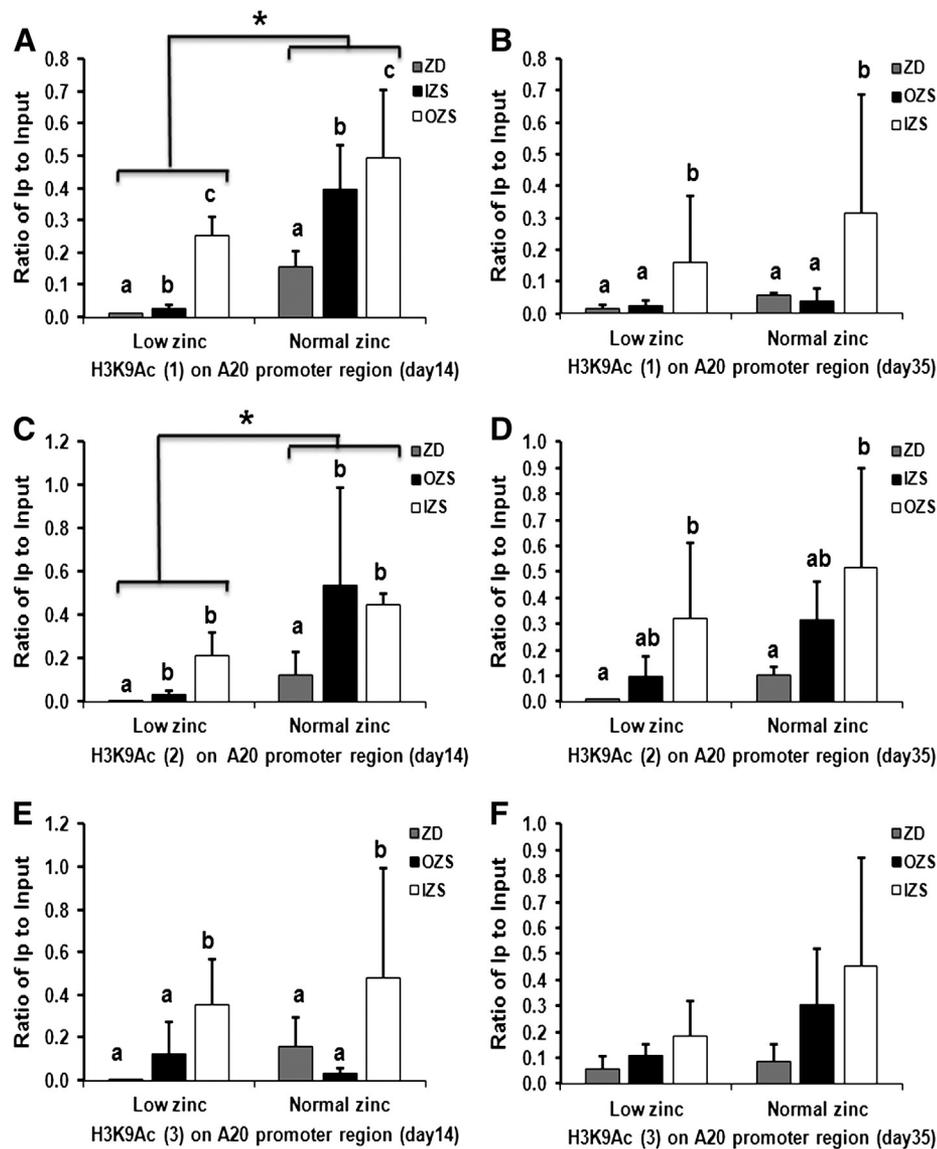


Fig. 9. Maternal and offspring Zn treatments suppressed H3K9 acetylation at the *A20* promoter. (A and C) Maternal and offspring Zn addition enhanced H3K9Ac at the promoter regions (340–425 and 506–603 bp) of the *A20* gene at day 14. (E) Dietary organic Zn supplementation in hens elevated the H3K9Ac levels of offspring jejunums (1691–1745 bp). (B, D and F) ChIP analysis of the *A20* gene in jejunal tissues at day 35 from the maternal and offspring Zn-treated groups using the antiacetylated H3K9 (A) antibodies. Levels of *A20* promoters in the IPs were measured by quantitative PCR. Statistical analysis was performed by SPSS GLM, and a one-way ANOVA was performed using the Duncan correction for multiple comparisons when the interaction was significant ($P < .05$). ^{a,b}Treatments with unlike letters were significantly different ($P < .05$).

unclear. Although our latest investigation in hens exhibited an increase in progeny jejunum weight after supplemental Zn in the broiler breeder diet compared with the maternal Zn-deficiency group (not published), the potential functional changes have not been clarified. In the current study, maternal Zn addition significantly increased villus height but decreased crypt depth in the progeny jejunums on days 14 and 35 compared with the Zn-deficient hens. Similarly, the offspring's jejunal mucosa function was enhanced, which was associated with elevated goblet cell counts per villus and the up-regulated mRNA expression of *MUC2* (Fig. 1) when hens were fed with an excessive Zn diet. Expectedly, organic Zn showed more improvement than inorganic Zn. *MUC2* secreted by goblet cells plays a pivotal role in keeping gut homeostasis through forming a nonspecific physical barrier [36]. However, *MUC2* knockout mice showed a decrease in intestinal epithelial barrier function [36,37]. Furthermore, there has been controversy related to the effects

between organic and inorganic Zn [38–41]. Thus, our results provide supportive evidence that supplemental organic Zn results in more attenuation of Zn-deficiency-induced intestine structure and function disorder compared with inorganic Zn treatment. sIgA is also essential for maintaining gut structure and epithelial barrier function [42]. Consistent with previous observations, sIgA production was markedly increased in the progeny jejunum mucosa from hens fed with 300 mg/kg organic Zn compared with the maternal Zn-deficient group. However, maternal inorganic Zn addition did not reverse the reduction of sIgA when offspring were fed with normal Zn diets. This may be partly due to the alleviated loss of sIgA after Zn supplementation in the offspring diet.

Measurement of proliferation and apoptosis has been documented to be a more reliable evaluation of the actual status of gut trophism [43]. We demonstrated that there was a 1.21-fold reduction of caspase 3 and a 1.57-fold elevation of PCNA in offspring jejunums from the hens that

received 300 mg/kg of organic Zn compared with the hens that received a low-Zn diet, suggesting that organic Zn is potentially beneficial for cell proliferation but not apoptosis. In addition, offspring dietary Zn levels could interact with maternal Zn status to affect the protein levels of PCNA and caspase 3 (Fig. 4). Taken together, these observations combined with enhanced gut mucosal barrier function are consistent with previous reports [44,45].

The immunoregulatory function of MUC2 has been demonstrated in intestinal epithelial cells by inhibiting NF- κ B-driven inflammatory signals [36]. Additionally, increased changes of proinflammatory factors have occurred in the Zn-deficient animals and cells [5,16,18,24,25]. Moreover, previous studies have shown that the inflammatory response is regulated by NF- κ B signaling [20]. Indeed, NF- κ B p65 mRNA levels in progeny jejunum on day 14 were obviously reduced by maternal and offspring dietary Zn addition (Fig. 5). Consequently, the transcription of *IL-1 β* , *TNF α* and *IL8* (Fig. 2) was repressed. Intriguingly, these changes, at least partly, persisted to day 35 (Fig. 3). Previous reports have indicated that loss of Zn in immune cells contributes to immune dysfunction and enhanced inflammatory response [29,46]. Recently, a novel zinc finger protein, A20, has been proven to be a critical negative regulator of NF- κ B signaling and inflammation [21,22,47,48]. A20 is essential for gut homeostasis and the normal regulation of innate and adaptive immune responses [21,49–51]. Moreover, Zn increases A20 induction, but the silencing of A20 enhances the generation of proinflammatory mediators TNF- α and IL-1 β [24,25]. To our knowledge, the present study is the first demonstration of the induction of A20 in Zn-supplemented avian animals (Fig. 5). Therefore, A20 may play a pivotal role in controlling gut inflammation and maintaining the mucosal barrier in birds.

Although maternal or offspring Zn deficiency triggered the NF- κ B-mediated inflammatory response and inhibited the expression of A20, previously there was no direct evidence of the relationship between Zn and A20. Of note, no difference of progeny jejunal Zn levels among maternal Zn treatments was found (data not shown). Furthermore, early nutrition and environmental conditions can alter epigenetic marks [52,53]. In addition, gene expression is susceptible to epigenetic changes resulting from alterations in early nutrition [54]. Moreover, Zn generates important effects on DNA methylation and histone modifications in mammals [28–30,46,55–57]. Interestingly, we found that the *de novo* methyltransferases DNMT3a and DNMT3b failed to display completely adverse results upon examination of A20 expression. Presumably, the maintenance DNA methyltransferase DNMT1 might play an equivalently important role in sustaining genomic methylation [58,59]. Nevertheless, global DNA hypomethylation was only observed after the maternal Zn treatment, not the offspring Zn treatment. In addition, A20 promoter methylation exhibited similar changes in global methylation status within the progeny jejunum. These observations, along with A20 mRNA levels, clearly indicate that Zn contributes to stimulating A20 transcriptional activity by suppressing global and A20-specific methylation. Furthermore, organic Zn enhanced the effects of inorganic Zn, which was associated with genomic and A20-specific demethylation. Together, our data support the evidence that transcriptional repression is closely related to DNA hypermethylation [60]. Additionally, a significant increase in DNA methylation frequency was observed in prenatal Zn-deficiency mice [30], which is in agreement with observed DNA hypermethylation in Zn-deficient oocytes [28]. Accordingly, the impaired genomic methylation states from maternal Zn deficiency can partially account for the repressed transcript of A20 and the loss of inflammation response. However, the offspring dietary Zn levels altered A20 mRNA levels without affecting DNA methylation status at day 14. Thus, it is conceivable that other epigenetic marks, such as histone acetylation, may be involved in this event.

It is clear that DNA methylation is interrelated with histone acetylation [60]. Moreover, histone acetylation is regulated by HATs

and HDACs. In the present study, HDACs were significantly down-regulated after maternal ZnMHA treatment at day 14 when progeny birds were fed with a normal-Zn diet. Unfortunately, we found no regular alterations in the HDACs. In contrast, maternal Zn exposure resulted in a moderate increase of HATs at day 14, which persisted to day 35. Therefore, we hypothesized that site-specific histone acetylation might exist. In fact, maternal Zn treatments failed to alter global H3K9Ac levels when offspring chicks were fed with a low-Zn diet. However, at the same time, maternal organic Zn supplementation increased H3K9Ac levels, and these changes persisted to day 35 in offspring that received dietary adequate Zn treatment. Consistent with A20 transcript levels, the H3K9Ac mark on the A20 promoter in maternal Zn-supplemented groups was higher than the maternal deficient-Zn group and site specific. Remarkably, H3K9Ac in the A20 promoter region (340–425 bp) showed a lasting increase in organic Zn-supplemented hens compared with the other two groups, whereas the Zn source failed to alter the levels of H3K9Ac in another promoter region (506–603 bp). Additionally, previous reports showed the site-specific alterations of epigenetic marks from early nutrition [30,54]. Most importantly, progeny birds that were fed an adequate Zn treatment displayed greater enrichment of H3K9Ac than those fed with a low-Zn diet at day 14. This may explain the enhanced transcription of A20 in offspring with normal Zn supplementation. Generally, these data appear to support previous demonstration that H3K9 is a critical epigenetic mark of gene activation [61].

In summary, our data highlight the role of A20 as a negative regulator of NF- κ B signaling. Maternal and offspring Zn exposure can potentially contribute to enhanced intestinal mucosal barrier function by promoting MUC2 transcription and sIgA production, repressing proinflammatory abundance and activating A20 expression. DNA hypermethylation and H3K9 hypoacetylation may be the important mechanisms behind A20 down-regulation in Zn-deficient birds. These findings imply that dietary Zn addition contributes to epigenetic alterations of A20 and later enhancement of innate immunity in the gut.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jnutbio.2014.10.005>.

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