

Prediction and Effect of Maternal Marginal Zinc Deficiency on Development, Redox Status, and Gene Expression Related to Oxidation and Apoptosis of Embryos

Wei Gao

South China Agricultural University

Liang Huang

South China Agricultural University

Xiufen Zhang

South China Agricultural University

Yaohui Zheng

South China Agricultural University

Wence Wang

South China Agricultural University

Wei Geng

Waterfowl Division, WENS Group

Chuang Liu

Waterfowl Division, WENS Group

Lin Yang

South China Agricultural University

Yongwen Zhu (✉ 408034085@qq.com)

South China Agricultural University <https://orcid.org/0000-0001-8605-4963>

Research

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Abstract

Background

Maternal severe zinc (Zn) deficiency induced oxidative damage and apoptosis in embryos, resulting in growth retardation. Therefore, it is crucial to assess maternal marginal Zn nutritional status for poultry breeders to prevent embryos subjected to severe Zn deficiency.

Methods

In Exp. 1, twelve egg embryos were sampled at incubation day 17 (E17), E20, E23, E26, E29, and E32 (day of hatch) respectively, with 6 replicates of 2 embryo each. The developmental changes of Zn mobilization and Zn transport gene mRNA expression were determined. In Exp. 2, 324 laying duck breeders were randomly allotted into 3 dietary Zn levels (0, 60, and 120 mg Zn/kg diet) with 6 replicates of 18 ducks per replicate. Plasma Zn concentration and erythrocytic Zn metalloenzyme activities in breeders as well as the development, redox status, and gene expression related to oxidation and apoptosis in embryos were measured. Blood samples were collected at the 2th, 4th and 6th weeks of the experiment.

Results

In Exp. 1, the overall Zn mobilization rates were increased in yolk sac and embryonic liver in response to the increased incubation period, associated with the decreased *ZIP10*, *13*, and *14* mRNA expressions in embryonic liver ($P < 0.05$). In Exp. 2, with the prolonged dietary Zn depletion, maternal Zn deficiency decreased plasma Zn concentration and erythrocytic alkaline phosphatase activity at the 6th week and inhibited erythrocytic 5'-nucleotidase (5'-NT) activity at 2th week ($P < 0.05$). On E29 of the maximal rate of Zn mobilization, maternal marginal Zn deficiency increased middle and late embryonic mortality and contents of superoxide anion radical, MDA and PPC, as well as decreased MT content, CuZnSOD activity, and *MT1* mRNA expression in embryonic livers ($P < 0.05$). Additionally, maternal marginal Zn deficiency increased *BCL2-associated X protein* and *Caspase-9* mRNA expression and decreased *B-cell lymphoma-2* mRNA expression in embryonic liver ($P < 0.05$).

Conclusion

Erythrocytic 5'-NT activity was more rapid and reliable to assess marginal Zn-deficient status in duck breeders. Marginal Zn deficiency impaired hatchability and antioxidant defense system and then induced the oxidative damage and apoptosis in embryonic liver, contributing to the greater loss of embryonic death.

Background

Zinc (Zn) is an essential trace mineral required for maintaining normal growth and development of embryos [1]. Maternal marginal Zn deficiency could lead to the susceptible of embryonic death predominantly [2]. Furthermore, severe Zn deficiency in maternal diets resulted in growth retardation,

abnormal development, and increased mortality of embryos [3]. Therefore, it is crucial to assess or predict maternal marginal Zn nutritional status to prevent embryos subjected to severe Zn deficiency. Some traditional and reliable of biochemical or functional indicators (e.g. tissue Zn contents and bone mineralization) have been proposed for estimating maternal Zn status [4]. In fact, only when Zn deficiency is relatively severe is it possible to detect changes in tissue Zn concentrations. It is necessary to select some specific sensitive biomarkers to predict maternal Zn status. Some studies in rat and human revealed that plasma Zn concentration could be used as a sensitive biomarker in response to Zn status [5, 6]. Alkaline phosphatase (ALP) is a Zn metalloenzyme and its activity in blood was decreased by 80% when Zn level was reduced from 96 mg/kg to 1.2 mg/kg in rat [7]. The 5'-nucleotidase (5'-NT) activity, as a cell membrane enzyme in erythrocyte and thymulin [8], was more sensitive to mild Zn deficiency than plasma Zn concentration [9, 10]. It is speculated that the activities of ALP and 5'-NT could be developed as specific sensitive biomarkers for predicting the marginal Zn-deficient status.

Maternal inadequate Zn supply decreased Zn deposition and then reduced the Zn mobilization from storage sites to the tissues of the developing embryos [11]. Thus, it is crucial to the survival of the embryo that the requisite amount of Zn would be available at the appropriate time during embryonic development. Zn as a cofactor of some distinct metalloenzymes, such as metallothioneins (MT) and copper-zinc superoxide dismutase (CuZnSOD), have a diverse range of biological reactions for maintaining embryonic development [12, 13]. Marginal Zn deficiency in maternal diet could lead to some adverse effects on antioxidant ability and anti-apoptosis during embryonic development [14]. In vivo study revealed that maternal dietary Zn supplementation could effectively eliminate chick embryonic mortality induced by maternal hyperthermia via enhancing antioxidant ability [12]. In rat, marginal Zn deficiency throughout gestation caused an induction of oxidative stress and impaired the normal development of fetal brain [15]. In vitro studies also have demonstrated that Zn deficiency in cell culture was conducive to the production of reactive oxygen (ROS) and the caspase activation [16]. In addition, Zn deficiency-induced apoptosis occurring was involved in a decrease in the AKT1 and ERK1 expression of growth factor signaling pathway during embryonic and fetal development [2]. It is hypothesized that a deficient in Zn availability can cause alterations in redox status and then lead to oxidative damage and cell apoptosis in tissue, contributing to abnormal embryo development.

In the present study, the developmental changes of Zn mobilization in embryos were investigated to determine the appropriate time of the maximal rate of Zn mobilization during middle and late incubation period. Then, we further studied the prediction and effect of maternal marginal Zn deficiency on embryonic development, redox status, and antioxidant and apoptosis related gene expressions using duck embryo model.

Methods

All procedures of our experiments were approved by animal care and welfare committee institute of South China Agricultural University.

Experimental design and diets

Embryonic development and Zn mobilization (Exp. 1) Fertile eggs (n = 72) from 33-wk-old female Muscovy duck breeders were obtained from a commercial breeder farm (WENS Group, Yunfu, Guangdong). Twelve egg embryos, representing the weight distribution of the eggs at set, were selected at incubation day 17 (E17), E20, E23, E26, E29, and E32 (day of hatch) respectively, with 6 replicates of 2 embryo per replicate. The egg, embryo, liver, yolk sac, and yolk-free embryo were weighed and the YSC was separated from the yolk sac, and then weighed and homogenized. The yolk sac and liver were stored at -20°C for Zn analyses. Small pieces of embryonic liver samples from E17, 23, and 29 were rinsed in a 0.9% autoclaved-saline solution and placed in microcentrifuge tubes at -80°C for analysis of mRNA expression of genes related to Zn absorption and transport.

Prediction and effect of maternal marginal Zn deficiency (Exp. 2). A total of 370 26-wk-old Muscovy duck breeders obtained from a commercial duck breeder farm (WENS Group, Yunfu, Guangdong) were housed in caged system for 4 wk period of adaptation. During the adaptation period, all ducks were fed restrictively with a commercial feed at the nutritional level (11.32 MJ metabolizable energy/kg, 180 g crude protein/kg, 7.0 g lysine/kg, 7.2 g methionine + cysteine/kg, 24.0 g calcium/kg, 3.8 g available phosphorus/kg, 40 mg Zn/kg). At 30 wk of age, 324 laying duck breeders were selected, balanced for laying rate and then randomly allotted into 3 dietary Zn levels with 6 replicates of 18 ducks per replicate. The experimental diets included 3 dietary supplemental Zn levels of 0 mg/kg (maternal Zn-deficient group, MZD), 60 mg/kg (maternal Zn-adequate group, MZA), 120 mg/kg (maternal Zn-high group, MZH) as inorganic Zn source. The basal diet was formulated to meet or exceed the nutritional requirements of laying duck breeders except Zn. The composition of the basal diet was shown in Table 1. The analyzed values of Zn contents in diets of MZD, MZA and MZH were 29.2, 63.4, and 163.4 mg/kg respectively. All birds had diets restriction and access to water ad libitum according to the operation manual and guideline of Muscovy duck breeders. The ducks received 16 h of daily lighting from 04:30 am to 20:30 pm. Room temperature and humidity were controlled by the air-conditioner and recorded daily. Manure was removed through an automatic belt system daily. All eggs were collected from each replicate daily and egg production (number of total laid eggs, defective eggs, and average qualified egg weight) were recorded daily. Feed consumption and egg weight were measured weekly. Feed intake was calculated by dividing total feed consumed by numbers of ducks per replicate per day. The eggs were incubated were incubated at a temperature of 37.8°C and a relative humidity of 55 to 60% for 28d. Eggs were candled at E7 and E28 to identify nonviable embryos. All removed eggs on E7 and E28 were counted, opened, and visually evaluated also to determine the true embryonic mortality.

Sample collections

The feed ingredients and diet samples from all the treatments were collected and analyzed for crude protein, calcium, phosphorus, and Zn contents. In each replicate cage, blood samples were collected via a bronchial vein from 2 fasted female duck breeders in each replicate on the last day of each period. Blood samples were separated into plasma and erythrocytes by centrifugation at at 3,000 × g for 15 min at 4°C.

Erythrocytes samples were washed three times in cold isotonic saline (0.9 %, v/w), then haemolyzed with a nine-fold volume of phosphate buffer (pH 7.4). The plasma and haemolyzed erythrocytes were stored at -20°C for further analysis.

Twelve eggs from each treatment (2 per replicate) were collected at the last day of 36 wk of age. The separated yolk from 2 eggs per replicate were pooled together and stored at -20°C for Zn analysis. On E29, 24 embryos (4 per replicate) from each treatment were killed by cervical dislocation. Equal weight sub-samples of the livers from the 4 embryos in each replicate were pooled into one sample for analyses. Total one gram liver sample of each replicate was homogenized at 8000 g for 10 sec in 9 mL of 0.9% sodium chloride buffer on ice and centrifuged at 3000 g at 4°C for 15 min, and the resultant supernatant was used for the analyses of antioxidant ability. The liver samples from the embryos were immediately dissected and frozen in liquid nitrogen and then stored at -80°C for further analyses of gene and protein expressions.

Determination of Zn concentration.

Zinc concentrations in samples including diets, plasma, egg yolk, yolk sac, and embryonic liver were measured using an inductively coupled plasma emission spectroscope (model IRIS Intrepid II, Thermal Jarrell Ash, Waltham, MA) after wet digestions with HNO₃ and HClO₄ as described by [11]. The total Zn contents of yolk sac and embryonic liver were calculated by multiplying Zn concentration and weight. The relative Zn mobilization rates were calculated as the ratio of Zn content in released Zn from yolk sac per day on average during E17-20, E20-23, E23-26, E26-29, and E29-32, respectively.

Determination of Zn metalloenzyme activities in erythrocytes

ALP activity was measured using a HITACHI 7180 automatic biochemical analyzer (Hitachi Ltd., Tokyo, Japan) with detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). CuZnSOD activity was determined by subtracting manganese superoxide dismutase (MnSOD) activity from total SOD (TSOD) activity according to the nitrite method [17]. 5'-NT activity was assayed by the determination of the P_i liberated from the substrate nucleotide as described by [18]. Total protein concentration in erythrocytes was determined using a BCA Protein Assay Kit (Cat no. 23225; Pierce). All indices of erythrocytes were expressed as nitrite units per milligram protein.

Determination of indices related to the oxidative damage

The activity of superoxide anion radical production was calculated and expressed as percentage of control (Vitamin C) based on the inhibition rate of superoxide anion radicals from the Xanthine and xanthine oxygenase reaction following the instruction of a commercial assay (A052-1-1, Nanjing Jiancheng Institute of Bioengineering, Jiangsu, China). The malondialdehyde (MDA) and protein carbonyl content (PCC) were determined by thiobarbituric acid colorimetric (A003, Nanjing Jiancheng Institute of Bioengineering) and 2, 4-dinitrophenylhydrazine methods according to kits (A087, Nanjing Jiancheng Institute of Bioengineering), respectively. The 8-hydroxy-2-deoxyguanosine (8-OHdG) was determined

using with a commercially available ELISA test kits (H165, Nanjing Jiancheng Institute of Bioengineering). All indices of supernatant were expressed as nitrite units per milligram protein.

Determination of antioxidant enzymes activities

Supernatant of the liver homogenization solution was used to measure the activities of glutathione peroxidase (GSH-Px) and catalase (CAT) using the commercial kits (Nanjing Jiancheng Bioengineering Institute) according to the instructions of the manufacturer. The total SOD and MnSOD activities were measured following the nitrite method described by Zhu et al. (2015), and CuZnSOD activity was calculated by subtracting MnSOD activity from total SOD activity. MT content was determined using a duck MT ELISA Kit (CG3309, Waltham, MA, USA).

RT-qPCR for gene mRNA expression

Total RNA was extracted from the embryonic liver tissues using Trizol reagent (Cat #15596018, Life Technologies) and then reverse-transcription was performed using QuantiTech Reverse Transcription Kit (Cat #205311, Qiagen) following the manufacturer's protocols with genomic DNA wiping off. The protocol of two-step PCR using ABI Power SYBR Green PCR Master Mix was conducted as described previously [17]. The primer sequences are listed in Supplementary Supplemental Table 1. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), was used to normalize the expressions of the targeted genes. The $2^{-\Delta\Delta C_t}$ was used to calculate mRNA level of each target gene.

Western blotting for protein expression

Total protein was extracted with ice-cold RIPA lysis buffer (Cat #P0013B, Beyotime Institute of Biotechnology, Haimen, China). The procedure following the preparation of the protein sample and SDS-PAGE, blotting transfer and detection of the protein specific antibodies were performed as described previously (26). The primary antibodies are listed in Supplemental Table 2.

Statistical analyses

Data of other indices were analyzed by one-way using the PROC GLM procedure of the SAS (SAS Inst. Inc., Cary, NC). Additionally, the significant effect of dietary Zn on plasma Zn concentration and Zn metalloenzyme activities in erythrocytes was analyzed for each week. The replicate served as the experimental unit. Differences among means were tested by the LSD method, and statistical significance was set at $P \leq 0.05$.

Results

Developmental change of embryos

The weights of duck embryo, embryo without yolk sac and embryonic liver increased linearly when incubation day increased, while the yolk sac weight decreased linearly as with the increased age ($P <$

0.0001; Fig. 1). The weights of embryo, embryo without yolk sac, and embryonic liver were increased from 32.84 g, 6.88 g and 0.057 g on E17 to 49.17 g, 45.67 g, and 1.188 g on E32, increased by 15.33 g, 38.79 g, and 1.13 g between E17 and E32, respectively. Between E17 and 32, the weight of yolk sac decreased from 26.7 g to 3.51 g.

Developmental change of Zn mobilization rate and Zn transport gene expressions

Zn concentration in yolk sac changed only slightly between E17 and E29 ($P > 0.05$) and declined significantly from E29 to E32 ($P < 0.05$; Fig. 2A). The Zn amount in yolk sac decreased linearly as with the increased incubation day ($P < 0.0001$; Fig. 2B). Between E17 and E32, total amount in yolk sac was declined from 1.092 g to 0.109 g, while Zn transfer out rate in yolk sac was increased from E20-23 to E29-32 (Fig. 2C). Between E20 and 29, Zn transfer in rate in liver was increased from E20-23 to E26-29, and then was decreased slightly by from E26-29 to E29-32 (Fig. 2D). Between E17 and 29, *MT1* mRNA expression in embryonic liver was increased with the increased incubation day, while *ZIP10*, *ZIP13*, and *ZIP14* mRNA expressions in embryonic liver was decreased with the increased incubation day ($P < 0.05$; Fig. 2E). The *ZIP8* mRNA expression in embryonic liver was increased from E17 to 23, and then decreased from E23 to E29 ($P < 0.05$). No difference on *ZIP6* mRNA expression was observed in embryonic liver with the increased incubation day during E17-E29.

Plasma Zn concentration and metalloenzyme activities in erythrocytes of breeder

Dietary Zn levels had no effect on CuZnSOD activity in erythrocytes of duck breeders at 32, 34 and 36 wk of age ($P > 0.05$; Fig. 3A). Dietary Zn deficiency decreased plasma Zn concentration (Fig. 3B) and erythrocytic ALP activity (Fig. 3C) of duck breeders at 36 wk of age, not at 32 and 34 wk of age ($P < 0.05$); whereas dietary Zn deficiency decreased erythrocytic 5'-NT activity of duck breeders at 32, 34 and 36 wk of age ($P < 0.001$; Fig. 3D).

Productive performance and zn concentration in egg yolk

Dietary Zn levels did not affect egg production performance of duck breeders during 30-36 wk of age, including egg weight, laying rate, egg mass and egg: feed ratio ($P > 0.05$; Fig. S1). Maternal dietary Zn levels affected Zn concentration in egg yolk, hatchability and middle and late embryonic mortality ($P < 0.05$; Fig. 4A-D), but did not influence chick hatch weight ($P > 0.05$; Fig. 4E). MZD group had lower Zn concentration in egg yolk and hatchability and higher middle and late embryonic mortality than MZH group, with no differences between MZD and MZA groups as well as between MZA and MZH groups.

Oxidative damage and antioxidant enzymes activities in embryonic livers

Maternal dietary Zn levels influenced ($P < 0.05$) the superoxide anion radical, MDA, PCC (Fig. 5A-C) and MT contents (Fig. 6A), GSH-Px (Fig. 6B) and CuZnSOD activities (Fig. 6E), but had no effect ($P > 0.05$) on the 8-OHdG content (Fig. 5D) and CAT activity (Fig. 6C) in embryonic livers on E29. Maternal dietary Zn deficiency increased the contents of superoxide anion radical, MDA and PPC and decreased GSH-Px

activity in embryonic livers. Breeders fed MZD diet had lower MT content and higher CuZnSOD activity in embryonic livers than birds fed MZH diet, with no differences between MZD and MZA groups.

Gene and protein expressions related to oxidation and apoptosis in embryonic livers

Maternal dietary Zn levels influenced *MT1*, *GPx*, *BAK-1*, *BAX* and *BCL2*, *Caspase-9* mRNA expressions ($P < 0.05$; Fig. 7), and had no effect ($P > 0.05$) on *CuZnSOD* and *CAT* mRNA expressions in embryonic livers on E29. MZH group had higher *MT1* and *BCL2* mRNA expressions and lower *GPx* and *BAK-1* mRNA expressions in embryonic livers than those from MZA and MZD groups, with no differences between MZA and MZD groups. MZA group had lower *BAX* and *Caspase-9* mRNA expression in embryonic livers than those from MZD and MZH groups, with no differences between MZD and MZH groups. Compared to MZD group, MZH group had higher signal protein expression of AKT1 and ERK1 in embryonic livers.

Discussion

Development and growth of poultry embryos are dependent upon the nutrient deposits in the eggs [19]. The yolk sac provides the chicken embryo with essential minerals nutrients for embryonic growth [20, 21]. Zinc is an essential nutrients required in small amounts for normal growth and development of the avian embryo functioning as catalytic or structural cofactors in metal-containing enzymes [1]. During incubation, approximately 86% of Zn originally present in the fertilized egg is transferred to the developing chicks. When Zn level was increased in the egg and yolk sac, the hatchability was increased primarily due to the decrease incidence of middle and later embryonic mortality [12]. Therefore, it is important to achieve an appropriate time of Zn mobilization exerting its biological functions to promote embryonic growth and development [22]. The developmental processes and Zn mobilization between yolk sac and liver tissue during the middle-later developmental stage of duck embryos have been studied in Exp. 1. In the current study, the weights of embryo, embryo without yolk sac and liver increased linearly as well as the weight of yolk sac decreased linearly between E17 and E32. A similar pattern was observed in chick embryos reported by [23, 24]. The Zn concentration in the yolk sac did not changed from E17 to E29 and only declined significantly from E29 to E32. It was indicated that close to hatch, the greater Zn demand may be required from yolk sac to the target tissues to maintain the rapid growth and development [25]. Also, our results showed that the calculated transfer out rate of Zn in yolk sac and transfer in rate of Zn in livers were both increased from E20-23 to E29-32. The parallel developmental changes of Zn mobilization rate in yolk sac and liver implied that the maximal rate of Zn mobilization on E29 could be conducted to serve a wide range of biological reactions, such as function as antioxidant defense system. In addition, the expressions of Zn transporters were sensitive to the changes of Zn status during embryonic development [26, 27]. The upregulation of *MT1* and downregulation of *ZIP10*, *ZIP13*, and *ZIP14* mRNA expressions were observed in embryonic liver on E29, which was confirmed that Zn homeostasis was strengthened on this appropriate time.

Many studies in rats and breeders have demonstrated that supplemental Zn is essential in diets to achieve normal reproductive performance [1, 16]. However, the results from our study indicated that

adding Zn to the diets had no effects on the characteristics of egg production performance of duck breeders, which was inconsistent with the positive results reported by in laying hens [28]. The differences between the studies may depend on the differences in the Zn sources, Zn content in basal diets, experimental periods and the genetic differences, age, and physiological states of the birds. However, feeding Zn deficiency in duck breeder diets resulted in a lower hatchability due to an increased middle-later mortality of embryos. The discrepancy between egg production and hatchability performance implied that embryonic development was much more sensitive to maternal Zn deficiency than egg production. It is also indicated that Zn requirement for laying performance might not be sufficient to maintain the hatchability and embryonic development of laying ducks. As reported previously, severe Zn deficiency in hen diets impaired egg production and embryonic development [29], whereas maternal Zn supplementation or in ovo injections of Zn in yolk can eliminate these adverse effects [30]. Therefore, it is necessary to assess Zn status measuring some specific sensitive indicators to prevent the marginal Zn deficiency occurred in breeder diets.

Some traditional and reliable of biochemical or functional indicators (e.g. tissue Zn contents and bone mineralization) have been proposed for estimating Zn status in poultry breeders [31]. In fact, only when Zn deficiency is relatively severe is it possible to detect changes in tissue Zn concentrations. Some studies revealed in rat and human that plasma Zn concentration and Zn metalloenzyme activities could be used as the sensitive biomarkers to permit estimation of the prevalence of marginal Zn deficiency [4]. For example, plasma Zn concentration is approximately 50 times lower than that in tissues, small differences in uptake or release of Zn from these peripheral sites can have a profound effect on the plasma Zn concentration. Studies in pregnancy of rat and human also found a significant increase in the plasma Zn concentrations following supplementation [5, 6]. In this study, MZD decreased the plasma Zn concentration and erythrocytic ALP activity of duck breeders at 36 wk of age but not at 32 and 34 wk of age. It is suggested that marginal Zn deficient status was occurred as with the prolonged dietary Zn depletion. Maternal dietary Zn deficiency decreased erythrocytic 5'-NT activity at 32, 34 and 36 wk of breeders. It is implied that erythrocytic 5'-NT activity responding to the Zn status was more rapidly and reliably and consequently possess the capacity to prevent the possible deleterious effects of severe Zn deficiency. Importantly, this further presses the need to establish the adequate biomarkers to ascertain Zn status, ultimately may shed light on human trials targeted Zn supplementation strategies to prevalence of marginal Zn deficiency in pregnancy.

Based on the appropriate time of Zn mobilization on E29, the effect of maternal marginal Zn deficiency on embryonic development was further studied. In the current study, maternal inadequate Zn decreased Zn deposition in yolk, implied that Zn supply to target tissues of the developing embryos could be declined. The liver is the most important organ for the storage and homeostatic regulation of Zn metabolism in the mobilization [25]. Zn as a cofactor of some distinct metalloenzymes, such as MT and CuZnSOD, were thought to be particularly important for maintaining Zn-dependent functions of antioxidant ability during embryonic development [12, 32]. We used the avian model to explore the effect of maternal marginal Zn deficiency on redox status in embryos during development. In this study, maternal marginal Zn deficiency decreased the ability of scavenging superoxide anion radical production

in association with the increase of MDA and PPC contents. Previous studies have reported that the excessive ROS from oxidative stress led to the damages of lipid and protein and then could arrest development of embryos [33]. Compared to maternal high Zn group, maternal marginally Zn-deficient group decreased the MT content and CuZnSOD activity in livers of duck embryos. Similar findings were reported for the developing chick embryo showing the consistency between hepatic Zn levels and redox state [25]. Thus, the oxidative damage of lipid and protein induced by marginal Zn deficiency related to the impaired antioxidant defense system could contribute to greater loss of the embryos. Furthermore, the parallel reduced *MT1* mRNA expressions were observed in the livers of embryos from breeder fed a maternal marginal Zn deficient diet. Many studies have demonstrated that MTs expressions correlated with hepatic Zn accumulation during development, and protects against the oxidative damage of Zn deficiency during pregnancy in transgenic and knock-out mice [34, 35]. In mice and chicken, it is proved that maternal Zn deficiency suppressed MTs mRNA expression of offspring embryos via epigenetic regulation by the DNA hypermethylation and histone hypoacetylation of gene promoter [12, 36]. Zinc deficiency has been shown to initiate apoptosis during development, altering embryogenesis [16]. In our study, there was greatly increased the mRNA expressions of *BAK-1*, *BAX* and *Caspase-9* in the liver of marginal Zn-deficient embryos, which are indicatives of cell apoptosis. For one thing, maternal marginal Zn deficiency might induce cell apoptosis by oxidative damage. A number of recent reports have been shown that enhanced expression of MT in cells induced anti-apoptotic effects. In other words, a lower MT content and expression in marginal Zn deficient embryos led to increased susceptibility of apoptotic cell death [37, 38]. In the current study, maternal dietary supplementation of 120 mg Zn/kg increased anti-apoptotic gene *BCL2* mRNA expressions in the embryonic liver to block or delay apoptosis, which was potentially in favor of embryonic development. In addition, a decrease in the AKT1 and ERK1 protein levels are observed in marginal Zn-deficient embryos. It is suggested that Zn deficiency-induced apoptosis was involved in growth factor signaling of AKT and ERK pathways by inhibiting cell cycle machinery [2].

Conclusions

Maternal marginal Zn deficiency impaired hatchability and increased middle-later embryonic mortality of duck embryos. Erythrocytic 5'-NT activity could be used as the sensitive biomarkers to assess Maternal marginal Zn-deficient status rapidly and reliably. On E29 of maximal rate of Zn mobilization, maternal marginal Zn deficiency impaired antioxidant defense system and induced oxidative damage and cell apoptosis in embryonic liver. These deleterious effects possibly contributed to greater loss of embryos during middle and late developing stage.

Declarations

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

YWZ and LY were responsible for all issues related to this paper. WG and LH were responsible for the planning of the study, sample collections, analyses, as well as the manuscript writing. YF, XFZ and WCW were involved in the sample collections, biological analysis, and statistical analyses. WG and CL were involved in the experimental design and data interpretations. All authors read and approved the final manuscript.

Availability of data and materials

Datasets obtained and analyzed in this research are included within this article (and the supplementary data files).

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Ethics approval and consent to participate

The study was reviewed and approved by the Institutional Animal Care and Use Committee at the College of Animal Science of South China Agriculture University, and carried out following the "Guidelines for Experimental Animal" of Ministry of Science and Technology (Beijing, P. R. China).

Consent for publication

All of the authors have approved the final version of the manuscript and agreed with this submission to the Journal of Animal Science and Biotechnology.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Composition and nutrient levels of the basal diets for laying duck breeders during 30-36 wk of age (as-fed basis)

Item (%)	Laying period
Corn	51.67
Soybean meal	17.70
Corn gluten meal	7.75
Wheat middlings	8.97
Lard	1.84
Dicalcium phosphate	1.80
Limestone	8.50
Sodium chloride	0.30
DL-Methionine	0.27
L-lysine·HCl	0.20
Vitamin and mineral premix ¹	1.00
Total	100
Nutrient composition	
Calculated value (%)	
metabolizable energy (MJ/kg)	11.63
Crude protein	18.51
Calcium	3.70
Total phosphate	0.60
Non-phytin phosphorus	0.44
Lysine	0.91
Methionine	0.57
Methionine + cysteine	0.84
Zinc ²	29.2

¹ Provided per kilogram of diet without Zn addition: vitamin A, 5, 000 IU; vitamin D₃, 800 IU; vitamin E, 20 IU; thiamine, 2.0 mg; riboflavin, 15 mg; pyridoxine, 4.0 mg; vitamin B₁₂, 0.02 mg; calcium pantothenate, 10 mg; folate, 0.15 mg; niacin, 60 mg; biotin, 0.20 mg; choline (Choline chloride), 1, 500 mg; Cu (CuSO₄·5H₂O), 8 mg; Fe (FeSO₄·7H₂O), 80 mg; Mn (MnSO₄·H₂O), 100 mg; Se (NaSeO₃), 0.3 mg; I (KI), 0.4 mg.

² Analysed values based on triplicate determinations.

Figures

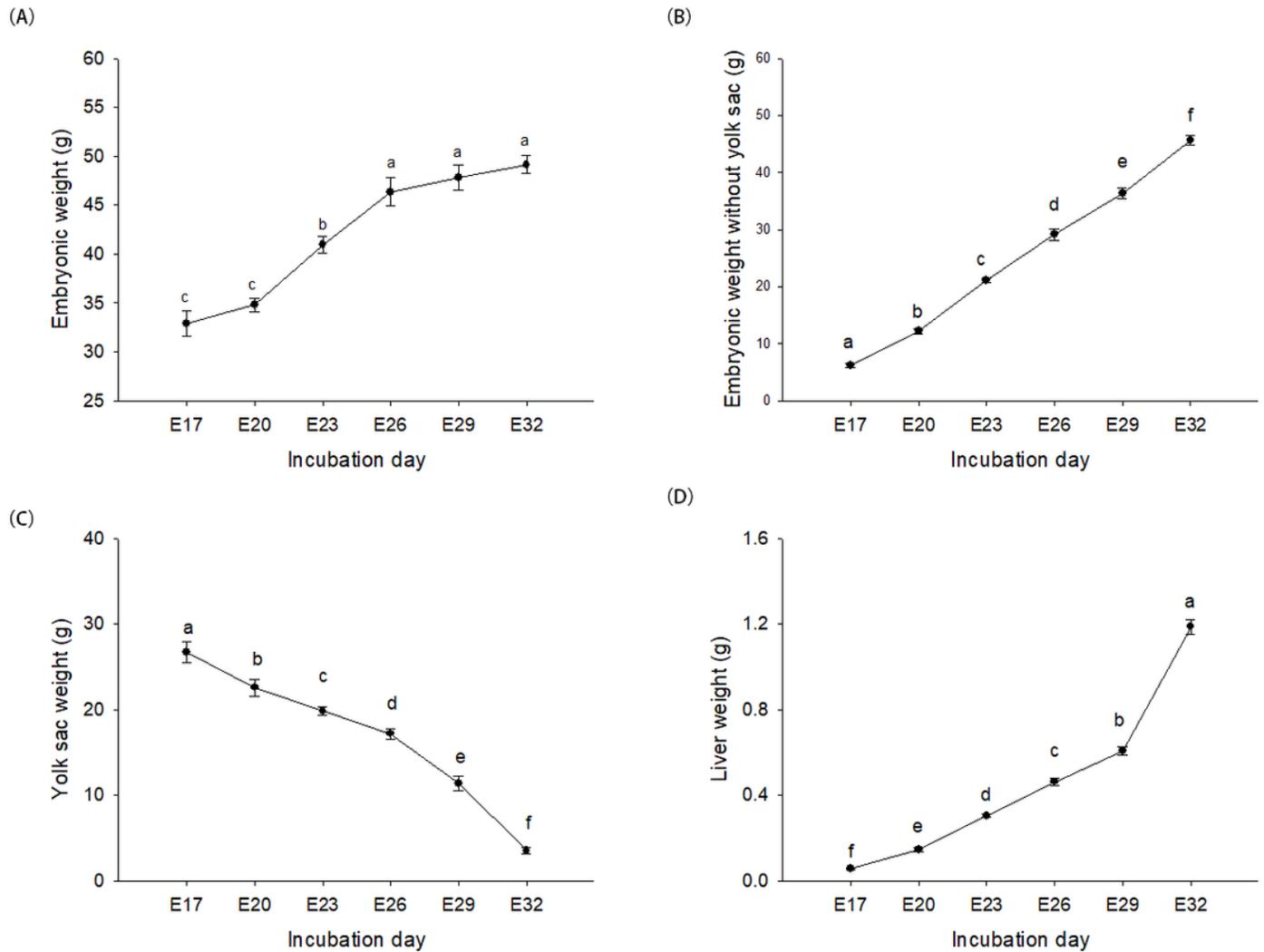


Figure 1

Developmental changes of weights in embryo (A), and embryo without yolk sac (B), yolk sac (C), and embryonic liver (D) weights during the middle and later incubation period (n = 10). All values are expressed as means \pm SE. Means with different letters (a-f) differ significantly ($P < 0.05$) between incubation days. MZD = maternal Zn-deficient group (0 mg Zn/kg diet); MZA = maternal Zn-adequate group (60 mg Zn/kg diet); MZH = maternal Zn-high group (120 mg Zn/kg diet).

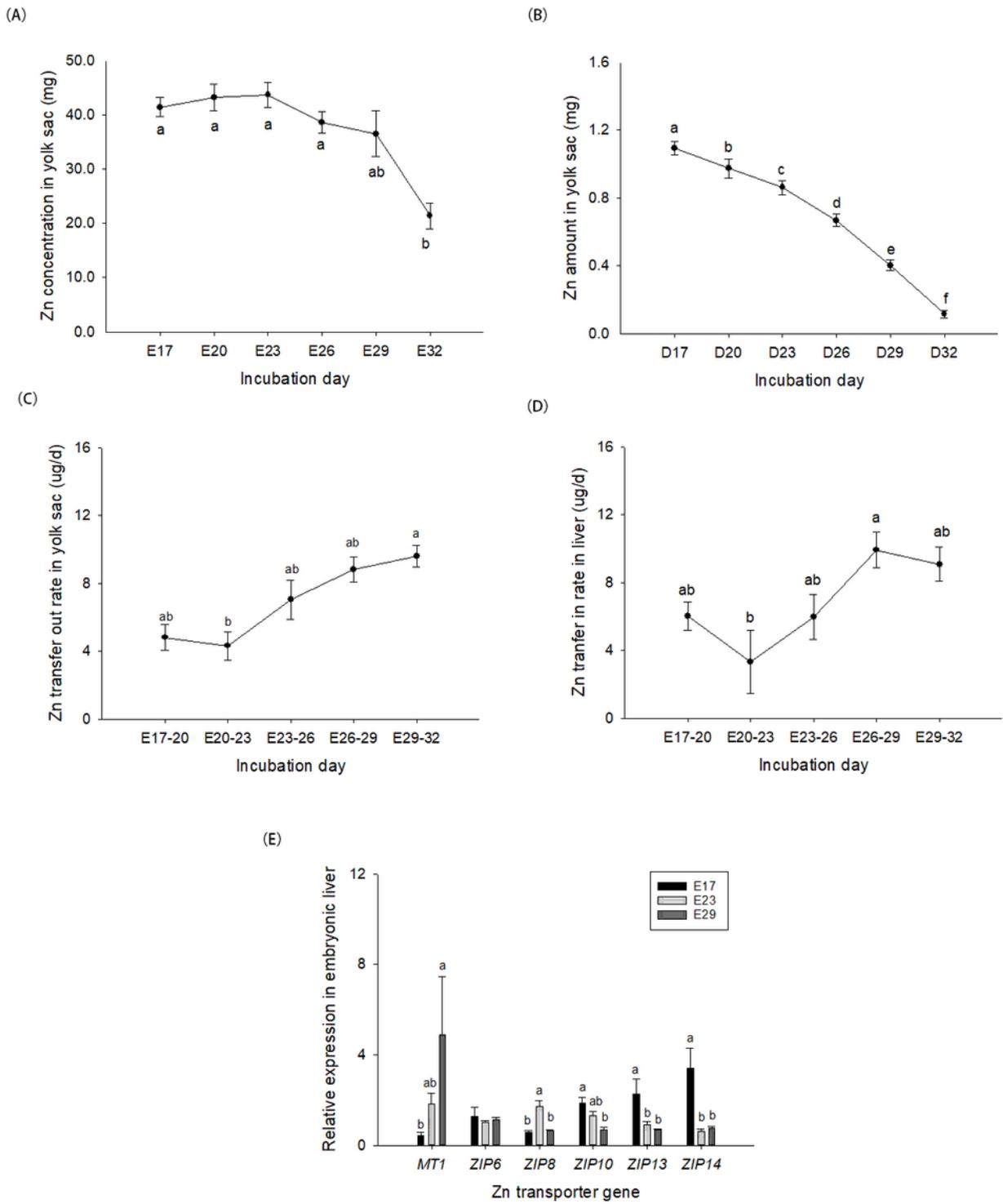


Figure 2

The developmental changes of Zn concentration (A) and Zn amount (B) in yolk sac, Zn mobilization rate of yolk sac (C) and liver (D), and Zn transporter gene mRNA expressions (E) in embryonic liver during the middle and later incubation period (n = 10). All values are expressed as means \pm SE. Means with different letters (a-f) differ significantly ($P < 0.05$) between incubation days. The total Zn contents of yolk sac and embryonic liver were calculated by multiplying Zn concentration and weight. Then, The relative Zn

mobilization rates were calculated as the ratio of Zn content in released Zn from yolk sac per day on average during E17-20, E20-23, E23-26, E26-29, and E29-32. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used to normalize the expressions of the targeted genes. MZD = maternal Zn-deficient group (0 mg Zn/kg); MZA = maternal Zn-adequate group (60 mg Zn/kg); MZH = maternal Zn-high group (120 mg Zn/kg); MT1= metallothionein 1; ZIP 6, 8, 10, 13, 14 = solute carrier family 39 member 6, 8, 10, 13, 14.

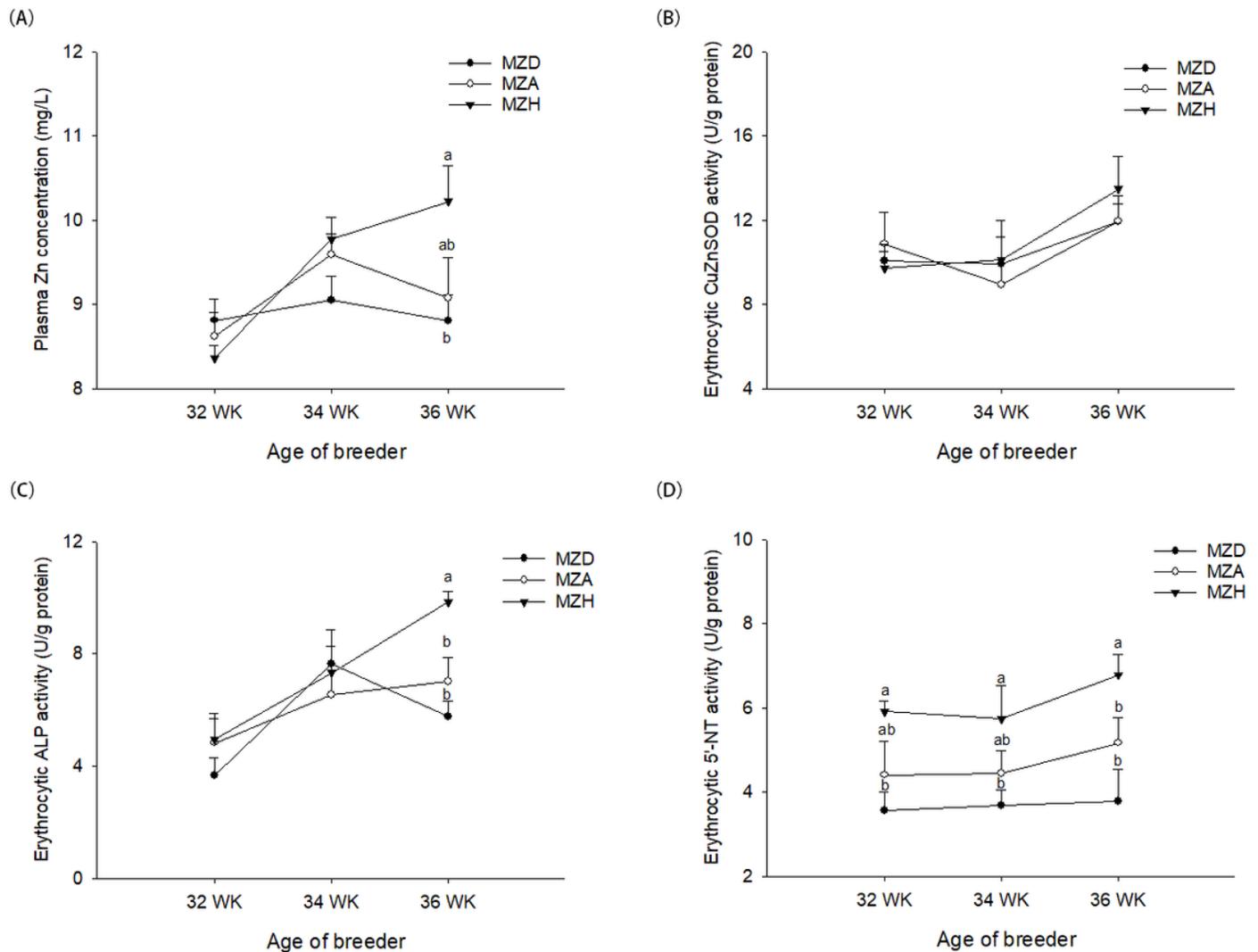


Figure 3

Effect of dietary Zn on plasma Zn concentration (A), and the activities of CuZnSOD (B), ALP (C) and 5'-NT (D) in erythrocytes of duck breeders at 32, 34 and 36 wk of age. All values are expressed as means \pm SE. The significant effect of maternal dietary Zn on plasma Zn concentration and Zn metalloenzyme activities in erythrocytes was analyzed for each week. Means with different letters (a-c) differ significantly ($P < 0.05$) between incubation days. MZD = maternal marginal Zn-deficient group (0 mg Zn/kg diet); MZA = maternal Zn-adequate group (60 mg Zn/kg diet); MZH = maternal Zn-high group (120 mg Zn/kg diet);

CuZnSOD = copper-zinc superoxide dismutase; ALP = alkaline phosphatase; 5'-NT = 5'-nucleotidase; WK = week.

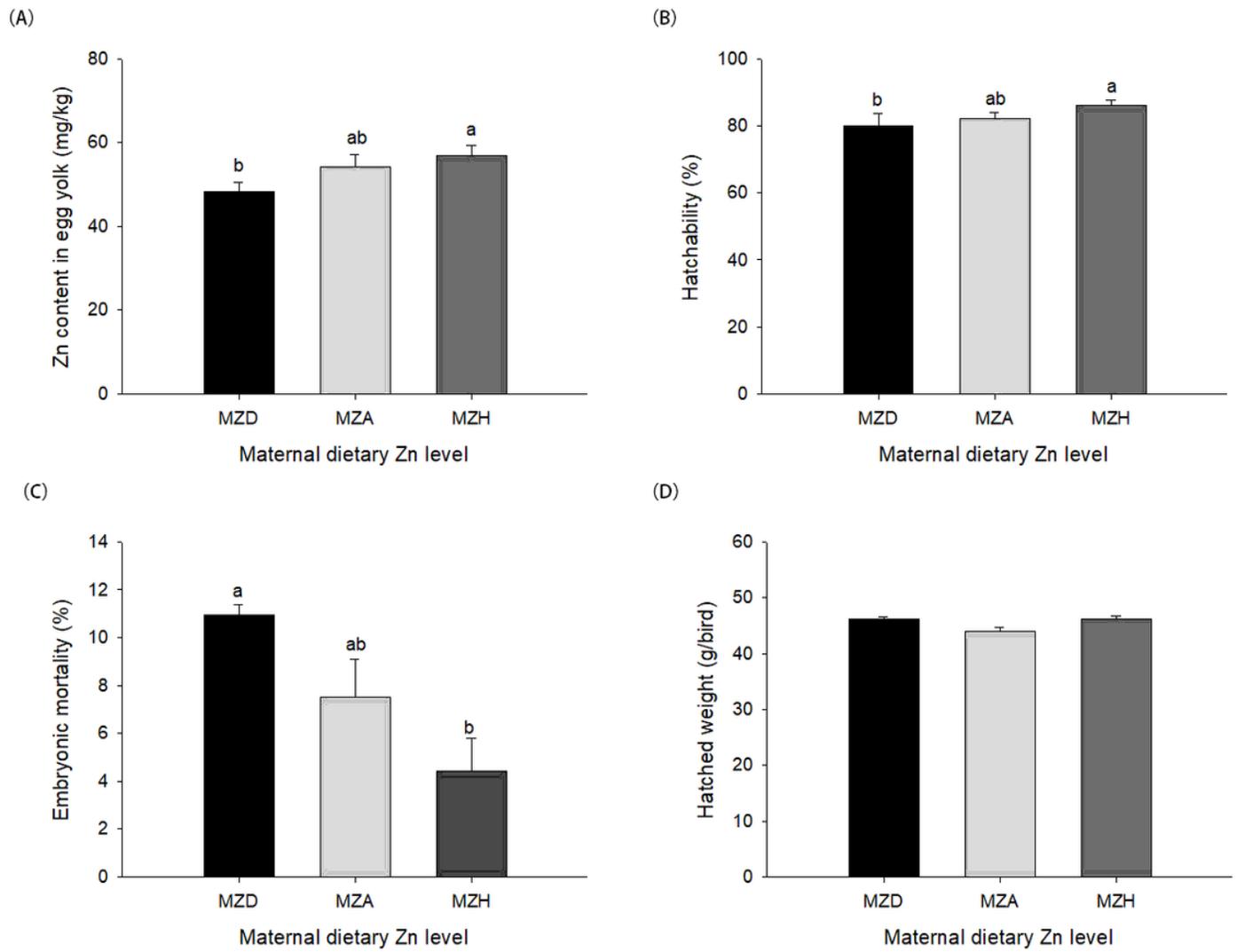


Figure 4

Effect of maternal dietary Zn on Zn concentration in egg yolk (A), hatchability (B), embryonic mortality (C), and chick hatch weight (D). All values are expressed as means \pm SE. Means with different letters (a-c) differ significantly ($P < 0.05$). MZD = maternal Zn-deficient group (0 mg Zn/kg diet); MZA = maternal Zn-adequate group (60 mg Zn/kg diet); MZH = maternal Zn-high group (120 mg Zn/kg diet).

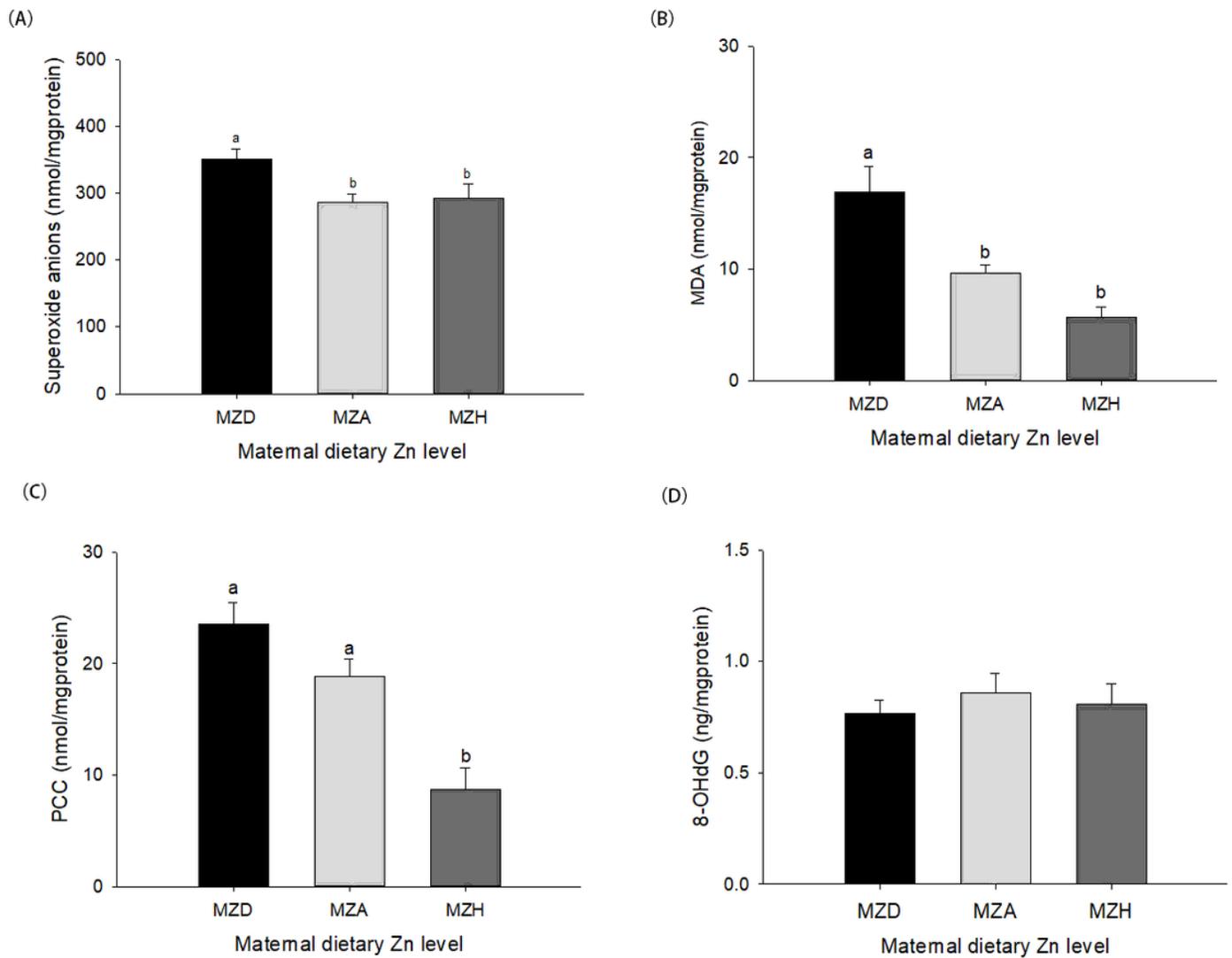


Figure 5

Effect of maternal dietary Zn on the contents of superoxide anion radical (A), MDA (B), PCC (C), and 8-OHdG (D) in embryonic livers at E29. All values are expressed as means \pm SE. Means with different letters (a-c) differ significantly ($P < 0.05$). MZD = maternal Zn-deficient group (0 mg Zn/kg diet); MZA = maternal Zn-adequate group (60 mg Zn/kg diet); MZH = maternal Zn-high group (120 mg Zn/kg diet); MDA = malondialdehyde; PCC = protein carbonyl content; 8-OHdG = 8-hydroxy-2-deoxyguanosine.

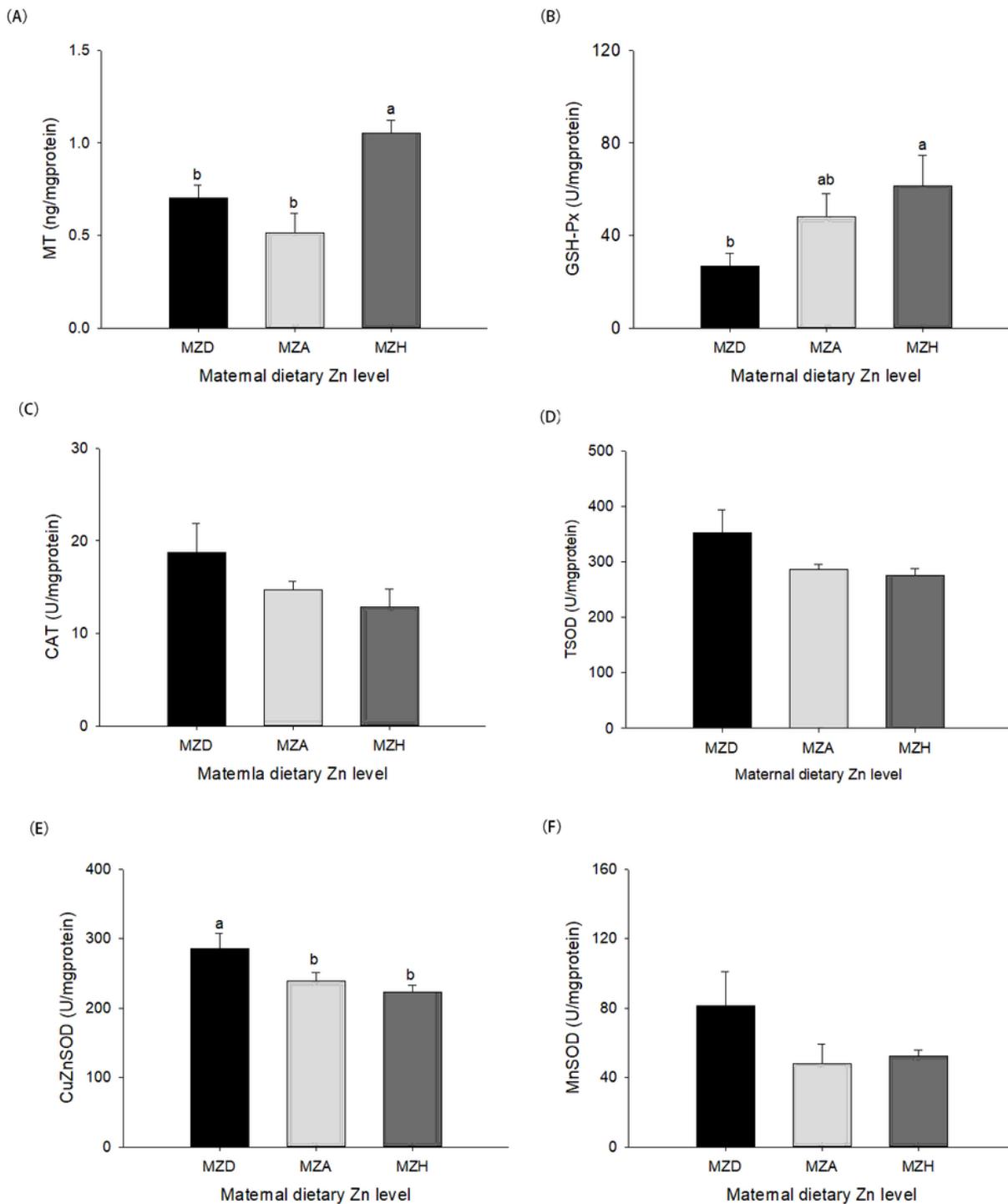


Figure 6

Effect of maternal dietary Zn on MT (A) content and GSH-Px (B), CAT (C), TSOD (D), CuZnSOD (E) and MnSOD (F) activities in embryonic livers at E29. All values are expressed as means \pm SE. Means with different letters (a-c) differ significantly ($P < 0.05$). MZD = maternal Zn-deficient group (0 mg Zn/kg diet); MZA = maternal Zn-adequate group (60 mg Zn/kg diet); MZH = maternal Zn-high group (120 mg Zn/kg

diet); MT = metallothionein; GSH-Px = glutathione peroxidase; CAT = catalase; TSOD = total superoxide dismutase; CuZnSOD = copper-zinc superoxide dismutase; MnSOD = manganese superoxide dismutase.

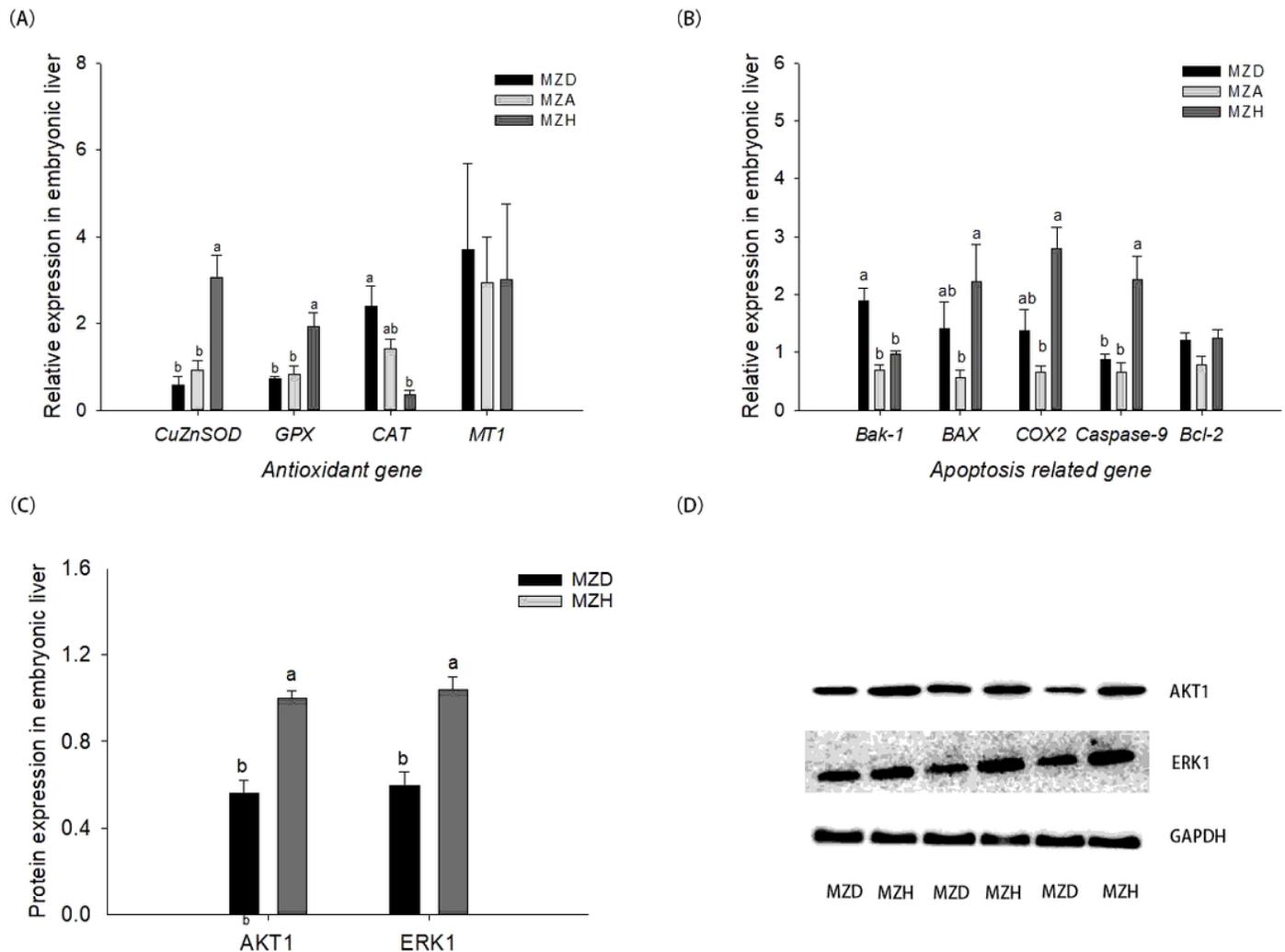


Figure 7

Effects of maternal dietary Zn levels on mRNA expressions of antioxidant gene (A) including CuZnSOD, GPx, CAT and MT1, and anti-apoptotic gene (B) including Bcl-2, Bak-1, BAX, COX2, and Caspase-9 as well as signaling AKT1 and ERK1 protein expression (C) including in the embryonic liver. Representative immunoblots of the indicated proteins were shown (D). The GAPDH mRNA expression was used to normalize the expressions of the targeted genes. The GAPDH was selected to normalize the expressions of target protein expression. Means with different letters (a-b) differ significantly ($P < 0.05$) between incubation days. MZD = maternal Zn-deficient group (0 mg Zn/kg diet); MZA = maternal Zn-adequate group (60 mg Zn/kg diet); MZH = maternal Zn-high group (120 mg Zn/kg diet); CuZnSOD = copper-zinc superoxide dismutase; GPx = glutathione peroxidase; CAT = catalase; MT1 = metallothionein 1; BCL2 = B-cell lymphoma-2; Bak-1 = BCL2 antagonist/killer 1; BAX = BCL2-associated X protein; COX2 = cyclooxygenase-2.

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