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The mRNA Expression of Host Defense Peptides in Chicken Erythrocytes are Highly Related to Tibial Dyschondroplasia and Induced by Recombinant Glutathione-S-Transferase A3 Protein

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Authors' Contribution
SN drafted the basic manuscript and analyzed the data. WXT and HCC contributed to conception and design of the research and reviewed the manuscript. ARJ, FJJ, XL, GBN, DZ, HLM and WFH participated in sample collection and laboratory testing. WWG, YJZ, SMG, GLL, JHL, FY and RKG participated in the data analysis and revising the manuscript.

Key words
Chicken, Erythrocyte, Glutathione-S-transferase A3, Host defense peptide, Tibial dyschondroplasia.

ABSTRACT

Chicken erythrocyte, which is the most abundant cell subset in blood vessel, also exists in cartilage zone. It has recently been shown that chicken erythrocytes constitutively express toll-like receptors (TLRs), cytokines, and avian β-defensins (AvBDs). Host defense peptides (HDPs) are important effector molecules in innate immunity. In this study, thiram has been used to induce tibial dyschondroplasia (TD) and the chickens were injected with different doses of recombinant glutathione-S-transferase A3 (rGSTA3) proteins. We explored the responses of HDPs in erythrocytes to thiram-induced TD and rGSTA3 protein by quantitative real time PCR (qRT-PCR). The results showed many HDPs expressions were suppressed by thiram-induced TD, and the expressions of HDPs were upregulated by rGSTA3 protein. These findings demonstrated that mRNA expressions of HDPs are highly related to thiram-induced TD, and rGSTA3 protein enhanced the immune response of chicken erythrocytes by up-regulating HDPs expression. Future studies may be aimed at elucidating the association between the expression of these peptides and the development of the TD.

INTRODUCTION

Tibial dyschondroplasia (TD) is a common group-occurring avian skeletal disease that is characterized by the accumulation of avascular cartilage in the metaphysis and can lead to varying degrees of lameness. For research purpose, thiram has been used to induce TD with high regularity and precision for young broiler chicks (Rath *et al*., 2007). By using thiram-induced model, some studies showed that vascularization had an important role in bone repair and pathology including TD, fracture healing, and rickets, *etc*. (Gay *et al*., 2007; Lee *et al*., 2012; Street *et al*., 2002; Zhang *et al*., 2013). The focus of early research was on the involvement of immune responses in bone diseases including TD (Nagahama *et al*., 2004; Philippart *et al*., 1993). For example, some research data indicated that low levels of several interleukins and macrophage expression of Galectin-3 (formerly called MAC-2 antigen) were detected during the development of bone lesions, and then returned to normal value after healing (Philippart *et al*., 1993). Moreover, other data showed that the deficiency of cytotoxic T lymphocyte-associated antigen 4 (*CTLA*-4) or programmed death-1 (*PD*-1), which can be induced on activated T cells, reduced osteoclastogenesis resulting in an osteopetrotic phenotype (Nagahama *et al*., 2004; Nabi *et al*., 2018).

In avian species, three main host defense peptides (HDPs) including avian β-defensins (AvBDs), cathelicidins (CATHs) and liver-expressed antimicrobial peptide-2 (*LEAP*-2) are important effector molecules in innate immunity and can kill various pathogenic broad-spectrum microorganisms (Butler *et al*., 2016; Cuperus *et al*., 2013; Van Dijk *et al*., 2008). Chicken erythrocyte, which is the most abundant cell subset in blood vessel, also

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exists in cartilage zone. Our group recently reported that the transcripts of eight AvBDs and *LEAP*-2 were found in chicken erythrocytes and they participated in MDV-induced host immune response (Niu *et al*., 2018). The transcripts of some types of toll-like receptors (TLRs) and cytokines were also detected recently in erythrocytes (St Paul *et al*., 2013). In addition, it has been shown HDPs are not confined to directly inhibit pathogen and can also control LPS-induced effects and promote phagocytosis, DNA uptake and wound healing (Cuperus *et al*., 2013; Semple *et al*., 2012; Zanetti, 2005). However, the biological role of the HDPs of chicken erythrocytes in TD has still not been explored.

Glutathione-S-transferases (GSTs), the main antidotal enzymes, have an important role in toxicology on account of its function that GSTs can catalyze the conjugation of nucleophilic GSH to various exogenous compounds (Townsend *et al*., 2003). Our study has previously identified that *GSTA*3 transcript was differentially expressed in chondrocytes after thiram-induced TD (Tian *et al*., 2013). GSTs also participate in the biosynthesis of classical PGs which can regulate chondrocyte differentiation and matrix synthesis (Chang *et al*., 1987; Li *et al*., 2004; O'Keefe *et al*., 1992). Acute cytotoxicity and genotoxic effects of aflatoxin B1 (*AFB*1) were reported to be more sensitive to *GSTA*3 knockout mice than the similarly treated wild-type mice (Ilic *et al*., 2010). These researches revealed that the rGSTA3 protein may have a potential role in the alleviation of TD.

In the occurrence of TD, a mass of nonviable cells has been produced in the cartilage, therefore, these cells must be removed before normal cartilage is restored. It has been recently shown that the tibial vascular distribution was suppressed after thiram-induced TD, and the levels of total RBC (red blood cell), HCT (hematocrit) and Hb (hemoglobin) were significantly reduced in the initial stage of TD (Huang *et al*., 2017). It has also been reported that vascularity within the developing growth plate can alleviate the clinical signs of TD (Street *et al*., 2002). These findings raise the possibility that immune responses of chicken erythrocytes also contribute to the prevention and treatment of TD. The objective of this study is to explore the effects of rGSTA3 protein and thiram-induced TD on the mRNA expression of HDPs in erythrocytes.

MATERIALS AND METHODS

Ethics statement

The management of the experimental animals was in agreement with the welfare guidelines approved by the College of Animal Science and Veterinary Medicine of Shanxi Agricultural University, China (Number 88, 2010). Before exsanguination and necropsy, injection of pentobarbital was used along with the standard protocols of euthanasia to minimize animals suffering.

Animals and reagents

One-day-old broiler chickens (n = 120) were bought from Shanxi Daxiang Farming Group (Shanxi, China) and raised in battery brooders, under a light regimen of 14 h light:10 h dark. A diet appropriate for their age was provided *ad libitum*. Thiram (JL131223002) was purchased from American Research Product (Massachusetts, USA); rGSTA3 protein was provided by College of Animal Science and Veterinary Medicine of Shanxi Agricultural University (Shanxi, China).

Induction of TD and rGSTA3 protein injection

From 1-day-old to 7-day-old chickens were fed a basal diet purchased from Shanxi Daxiang Farming Group (Shanxi, China). On days chickens were divided randomly into 6 groups (groups A–F, 20 chicks/group). Groups A, B and C were the basal diet groups, and groups D, E and F were the thiram-fed groups. Starting from day 8, the thiram-fed groups (D, E and F) were fed a diet containing 100 mg/kg of thiram for 2 days to induce TD, and then the chickens in all 6 groups were fed basal diet. On day 8, 10, 12, and 14, 4 treatment groups (B, C, E, F) were injected rGSTA3 proteins at a dosage of 20 μg/kg (groups B and E) and 50 μg/kg (groups C and F) 4 times, as previously described (Wang *et al*., 2018). On day 12 and 23, the blood samples were collected.

Blood collection and erythrocyte isolation

Blood samples were drawn from wing vein (2 mL per bird) and collected into anticoagulant tubes, and then diluted at 1:1 in Alsever's solution. The diluted blood was overlaid onto Histopaque solution (Histopaque-1119; Sigma-Aldrich, USA) and then centrifuged at 500g for 20 min. Subsequently, the platelets and leukocytes-containing supernatant were removed as previously described (St Paul *et al*., 2013). Erythrocytes isolated were determined to be > 99.9% pure by Wright–Giemsa staining.

RNA extraction and cDNA synthesis

Total RNA was purified from erythrocytes isolated from blood samples by using RNAiso Plus (Takara Bio Inc., Dalian, China) according to an improved method and then dissolved in 20 μL DEPC-treated water. The preliminary quantity and purity of the extracted RNA was measured by a NanoDropBioanalyzer ND1000 (Labtech, Uckfield, UK) and RNA integrity was verified by 1.5% agarose gel electrophoresis. Total RNA (500 ng) was reverse transcribed to cDNA using the reverse transcription

kit (PrimeScript RT reagent Kit; Takara Bio Inc., China) according to the manufacturer's recommended protocol. The cDNA samples were diluted at 1:10 in DEPC-treated water and stored at -20°C.

Analysis of mRNA transcripts by quantitative real time PCR (qRT-PCR)

qRT-PCR was performed using a quantitative PCR kit (TaKaRa SYBR Premix Ex Taq™ II; Takara Bio Inc., China). The primers of HDPs were designed by Primer Express 3.0 according to the coding sequences of the respective chicken genes in the National Center for Biotechnology Information database and synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China). Detailed information for the primers of HDPs and the annealing temperature set in this experiment are presented in Table I. The thermal cycling parameters were used for qRT-PCR as previously described (Wang *et al*., 2018). Expression levels of HDPs relative to the housekeeping gene 18SrRNA were determined with the QuantStudio™ 6 Flex Real-Time PCR System Software (Applied Biosystems, USA).

Statistical analysis

The qRT-PCR data was analyzed using the $2^{-\Delta\Delta Ct}$ method. The results represent mean fold change from the control group A ± standard error. The significance of differences among different groups was analyzed using SPSS Statistics 17.0 (IBM Company, New York, NY, USA). The data of qRT-PCR was used by one-way ANOVA to identify treatment differences which were considered significant when $P < 0.05$.

RESULTS

Reproduction of the thiram-induced TD

A dramatic increase of TD chickens was observed on day 12 and then the clinical signs of TD chickens significantly recovered on day 23, and the clinical signs and pathological characteristics of thiram induced TD has been showed in our recently published research (Wang *et al*., 2018).

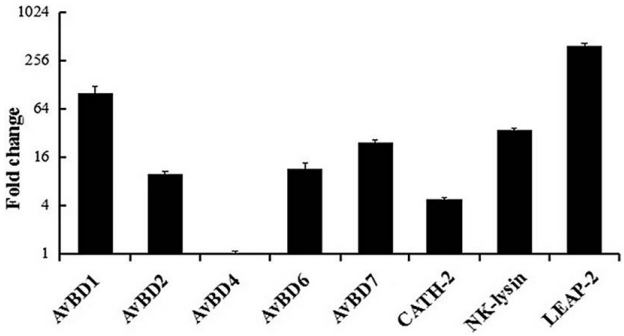

Fig. 1. Expression pattern of HDPs transcripts in the un-stimulated erythrocytes of 23-d-old broilers relative to the house-keeping gene 18SrRNA.

Table I.- Primer sequences and reference genes used for quantitative real-time PCR.

Target genes	Primer sequence (5′→3′)	Annealing temp.	GenBank accession number
AvBD1	F: CCGTTTCTGTCACCGTCA R: CCTTTGCTAAAAATCCCTTC	57°C	NM_204993.1
AvBD2	F: GCACTCCAGGTTTCTCCA R: GGCGTCCGACTTTGATTA	57°C	DQ677633.1
AvBD4	F: TCATGGAGCTGTGGGCTTTT R: AGCATTCCCATAAGGGCATT	55°C	NM_001001610.2
AvBD6	F: CTGCTGCTGTCTGTCCTCTT R: TGCAGACACCCCTTTGATAT	55°C	NM_001001193.1
AvBD7	F: CTATTGATACTTGTTGGCTTCG R: AACTCCTCCATCCCCTTG	57°C	AY621322.1
CATH-2	F: GACGACTGCGACTTCAAGGA R: CGTCTCTGCAGCGTAGATTG	55°C	KT962965.1
NK-lysin	F: TTCTGCGTCAGTCTGGTGAA R: TCCCCGTACTGCACACCTT	55°C	KT962967.1
LEAP-2	F: ACTCTGGAATTCTGCCTGATGACA R: CATCTGCATCCGTGCCTGA	57°C	NM_001001606.1
18SrRNA	F: TTCCGATAACGAACGACAC R: GACATCTAAGGGCATCACAG	55°C	FM165414

Erythrocytes constitutively express many HDPs at the transcript level

To determine the repertoire of HDPs expressed in chicken erythrocytes, the transcript levels of HDPs in erythrocytes of control group (group A) was assayed by using qRT-PCR (Fig. 1). The results showed that erythrocytes constitutively expressed transcripts for *AvBD* 1, 2, 4, 6, 7, *CATH*-2, *NK-lysin* and *LEAP*-2. Among the transcripts, the *LEAP*-2 was the most abundant while the AvBD4 was least abundant.

Effects of rGSTA3 protein and thiram-induced TD on the mRNA expression of HDPs in erythrocytes

The gene expression patterns of HDPs in erythrocytes were quite different among the groups. The effects of the rGSTA3 protein and thiram-induced TD on the expression of HDPs in erythrocytes on day 12 and 23 are shown in Figures 2 and 3. In 12-day-old birds, stimulation with 50 μg/kg rGSTA3 protein resulted in the significant upregulation of 6 types of HDPs (*AvBD*1, 2, 4, 6, 7 and *LEAP*-2) in rGSTA3-injected birds compared with those from the control group ($P < 0.05$). The transcripts of *AvBD*1, 4 and 6 were significantly upregulated by 50 μg/kg rGSTA3 protein in thiram-treated birds and control birds ($P < 0.05$). The expression of *LEAP*-2 mRNA was upregulated ($P < 0.05$) by thiram. On the contrary, thiram significantly reduced the expression of *AvBD*2, *AvBD*7, *CATH*-2 and *NK-lysin* in 12-day-old birds ($P < 0.05$). In 23-day-old birds, the expression of *AvBD*1, *AvBD*2, *AvBD*7, *CATH*-2 and *LEAP*-2 showed a dose-dependent pattern in response to rGSTA3 protein in thiram-fed groups. Moreover, thiram significantly reduced the transcripts of *AvBD*1, 2, 4, 6, 7, *LEAP*-2 and *NK-lysin* ($P < 0.05$). In addition, after the 50 μg/kg of rGSTA3 protein stimulation, the highest upregulation was detected for *NK-lysin* with 60.2-fold higher expression compared with the control group ($P < 0.01$).

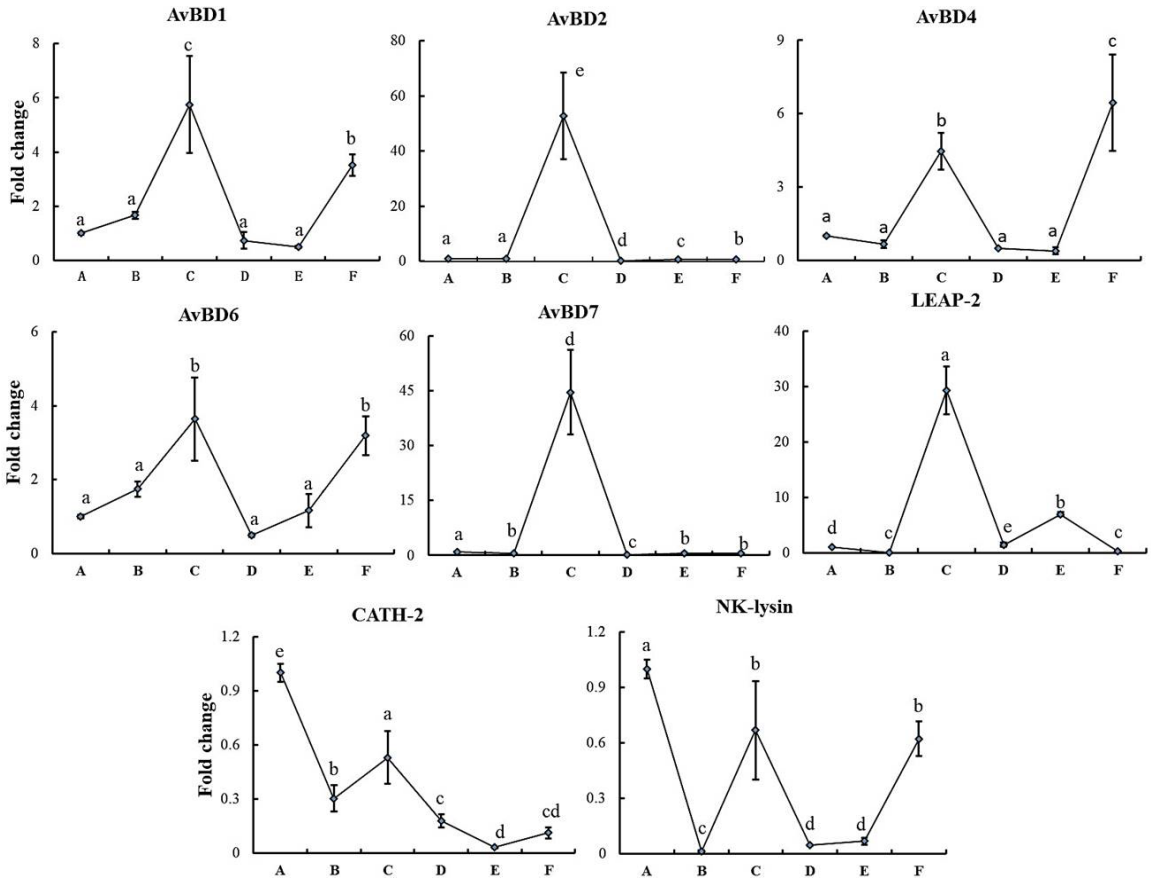

Fig. 2. The changes in the expression levels of mRNAs of HDPs in chicken erythrocytes of 12-day-old broilers. Basal diet groups (A, B and C) treated with 0, 20, 50 μg/kg of recombinant glutathione-S-transferase A3 (rGSTA3) protein, and thiram-containing diet groups (D, E and F) treated with 0, 20, 50 μg/kg of rGSTA3 protein. In each panel, different lowercase letters (a–e) indicate statistically significant differences ($P < 0.05$).

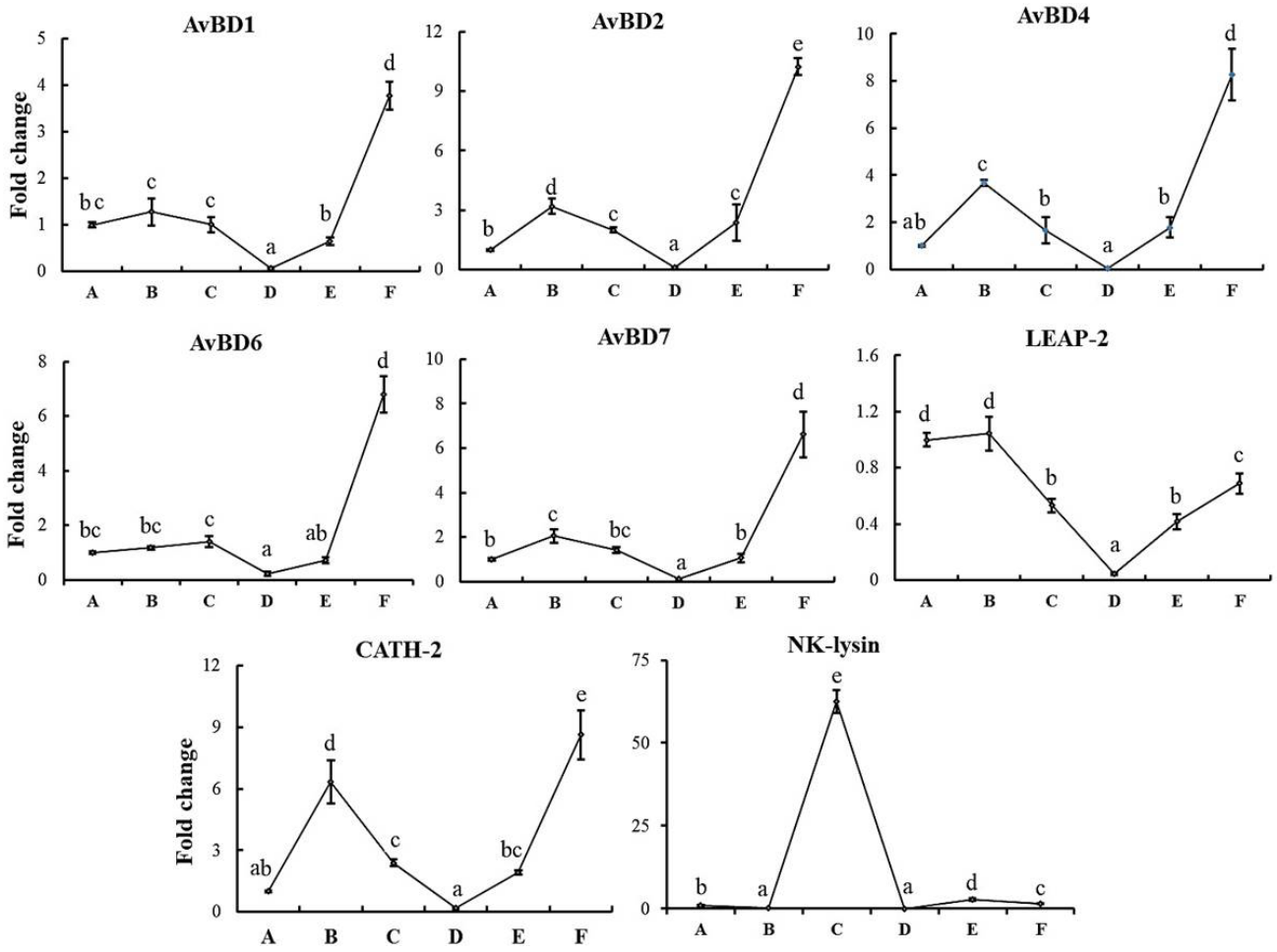

Fig. 3. The changes in the expression levels of mRNAs of HDPs in chicken erythrocytes of 23-day-old broilers. Basal diet groups (A, B and C) treated with 0, 20, 50 μg/kg of recombinant glutathione-S-transferase A3 (rGSTA3) protein, and thiram-containing diet groups (D, E and F) treated with 0, 20, 50 μg/kg of rGSTA3 protein, respectively. In each panel, different lowercase letters (a–e) indicate statistically significant differences ($P < 0.05$).

DISCUSSION

TD is an avian pervasive disease, in which growth plate cartilage accumulates in the metaphyseal region of the tibiotarsus and chondrocytes a large number of nonviable cells that can cause inflammation (Orth and Cook, 1994). Recent studies have reported that erythrocytes have a direct role in the immune response and can produce many cytokines and AvBDs in response to some pathogens and proinflammatory factors (Morera and MacKenzie, 2011; St Paul *et al*., 2013). It has also been shown that tibial angiogenesis in the hypertrophic zone was strongly suppressed by thiram-induced TD (Huang *et al*., 2017). These clues suggested that the development of TD may be associated with the immune response of chicken erythrocytes in the blood vessels. In this study, we explored the effects of rGSTA3 protein and thiram-induced TD on the expression of HDPs in erythrocytes. Here, we found chicken erythrocytes constitutively express many HDPs transcripts and the expression of HDPs has remarkable changes after stimulation of rGSTA3 protein and thiram-induced TD.

In the current study, the results indicated that erythrocytes constitutively expressed transcripts of AvBDs, *LEAP*-2, *CATH*-2 and *NK-lysin* with the transcript of *LEAP*-2 being the most abundant. Previous report showed that *LEAP*-2 was highly expressed in the chicken liver and human blood (Krause *et al*., 2003; Lynn *et al*., 2003). The data indicated that HDPs especially *LEAP*-2 may participate in the immune response of many diseases including TD. In addition, the results of this study showed that thiram-induced TD significantly upregulated the

expression of *LEAP*-2 mRNA, but significantly reduced the expression of *AvBD*2, *AvBD*7, *CATH*-2 and *NK-lysin* in 12-day-old birds. The results suggested that thiram-induced TD induced the synthesis of *LEAP*-2 and disturbed the expression of *AvBD*2, *AvBD*7, *CATH*-2 and *NK-lysin*. At 23-day-old, thiram-induced TD not only significantly reduced the transcript of *NK-lysin*, which is similar to 12-day-old birds, but also significantly decreased the transcripts of *AvBD*1, 2, 4, 6, 7 and *LEAP*-2. These results evidenced that thiram-induced TD can lead to a certain extent damage of the immune system with the suppressed immune function of many HDPs. Similarly, it was recently reported that antiapoptotic proteins in erythrocytes were suppressed in the initial stage of TD and induced in TD recovery (Wang *et al*., 2018). Further, it has been showed that the angiogenesis can be inhibited by thiram-induced TD, and the occurrence of TD can be caused by the angionecrosis in growth plate (Marikovsky, 2002; Rath *et al*., 2005). In the present study, the transcripts of 6 types of HDPs (*AvBD*1, 2, 4, 6, 7 and *LEAP*-2) were significantly upregulated by stimulation with 50 μg/kg rGSTA3 protein in 12-d-old birds, but were recovered to the normal level at 23 d old. These results showed that high level of rGSTA3 protein can induce *AvBD*1, 2, 4, 6, 7 and *LEAP*-2 expression to enhance immune response in initial stage of TD, but their expression restore to the original level in the recovery phase of TD. Moreover, our group has recently reported that AvBDs in chicken erythrocytes may participate in MDV-induced host immune responses (Niu *et al*., 2018). At 12-day-old, the transcripts of *AvBD*1, 4 and 6 were significantly upregulated by 50 μg/kg rGSTA3 protein in thiram-fed birds and basal diet birds; *AvBD*1, 2, 7, *CATH*-2 and *LEAP*-2 showed a dose-dependent expression in response to rGSTA3 protein in thiram-fed groups at 23-day-old. These results suggest that the recombinant protein could initiate a vigorous immune response by stimulating the expression of various HDPs in erythrocytes. Similarly, we have also reported that rGSTA3 protein participated in the recovery of TD by promoting the expression of apoptosis-related genes in erythrocytes (Wang *et al*., 2018). *NK-lysin*, a member of HDPs, was generally considered to be produced by cytotoxic T cells and natural killer cells (Peña *et al*., 1997). It has diverse immunomodulatory capabilities, such as the modulation of pro-inflammatory and anti-inflammatory responses, angiogenesis, wound-healing and apoptosis (Hilchie *et al*., 2013; Kim *et al*., 2017; Mansour *et al*., 2014). In the current study, after 50 μg/kg of rGSTA3 stimulation, the highest upregulation was detected for *NK-lysin* with 60.2-fold higher expression in 23-day-old compared with the basal diet-fed controls. The result showed that *NK-lysin* transcript also existed in chicken erythrocytes, and it is possible that rGSTA3 protein promotes the regulation of growth plate vascularization and chondrocyte maturation by stimulating the expression of *NK-lysin* in erythrocytes. As of yet, it is still not very clear as to what the biological function of HDP-stimulated erythrocytes is during the development of TD. There is one possible immunological role for HDPs in chicken erythrocytes that toll-like receptors (TLRs) recognize some proinflammatory factor produced in the process of TD, and then induce intracellular signals through NF-kB-dependent pathways to induce HDPs and cytokine expression that enhance the phagocytic activity (St Paul *et al*., 2013; Zarember *et al*., 2002).

CONCLUSION

The expression of HDPs transcripts is very different in chicken erythrocytes. In the development of TD, many HDPs expression were suppressed by thiram-induced TD, and rGSTA3 protein promoted the immune response by up-regulating the expression of HDPs. which suggested that these HDPs expression is highly related to thiram-induced TD, and rGSTA3 protein can induce the expression of all HDPs to promote the recovery of TD. Further studies are needed to elucidate the mechanism of HDPs in response to the rGSTA3 protein as well as thiram-induced TD, and the association between the expression of these peptides and the development of the TD.

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Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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