Pakistan J. Zool., vol. 56(2), pp 817-825, 2024 DOI: https://dx.doi.org/10.17582/journal.pjz/20200603180624

Effect of Recombinant Glutathione-S-Transferase A3 Protein on the Expression of Host Defense Resistance and Pattern Recognition Receptor in the Thiram Induced Tibial Dyschondroplasia Chicken Erythrocytes

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Article Information
Received 03 June 2020
Revised 22 October 2020
Accepted 10 November 2020
Available online 09 January 2023 (early access)
Published 06 February 2024

Authors' Contribution
WXT conceived and designed the experiments. RAM, DZ, SXY, MHM and SN analyzed the data. ARJ, MLQ, XYH, YW and MFQ performed the experiments. RAM wrote the paper. RAM, ARJ and WXT revised the manuscript.

Key words
Immunity, Interferon regulatory factor, Thiram, Tibial dyschondroplasia, TLR pathway

ABSTRACT

Tibial dyschondroplasia (TD) is one of the most common problems in broiler chickens. Chicken erythrocyte is the most abundant cell found in blood vessels, in which host defense resistance (HDR) and pattern recognition receptors (PRRs) play a significant role in the immune response. However, the function of HDR and PRRs in thiram induce TD chicken is not fully known yet. Therefore, this study was designed to evaluate the effect of recombinant glutathione-S-transferase A3 (rGSTA3) protein on HDR and PRRs in the thiram-induced TD chicken. One hundred twenty arbor acres (AA+) broiler chickens of seven-day-old were equally divided into six groups. Group A, B, and C were treated with 0, 20, 50 $\mu g \cdot kg^{-1}$ of rGSTA3 protein, respectively. Group D, E and F were treated with 0, 20, 50 $\mu g \cdot kg^{-1}$ of rGSTA3 protein + thiram 100 $\mu g \cdot kg^{-1}$. The results showed that chicks treated with thiram showed a higher TD score with typical lesions in the proximal growth plate than the rGSTA3 supplementary group. A morphological characteristic of TD was measured by TD index. Chicken erythrocytes constitutively expressed transcripts for HDR and PRRs genes. Moreover, the quantitative real time PCR (qRT-PCR) results showed that HDRs and PPRs expressions were suppressed by thiram, which found upregulated in rGSTA3 protein supplementary group. These findings demonstrated that mRNA expressions of HDRs and PPRs are highly related to thiram. Moreover, rGSTA3 protein increased the immune response of HDRs and PPRs. Conclusively the results of this study provide the strong evidence that rGSTA3 protein can promote an immune response in chicken erythrocytes by up regulating the expression of HDR and PRRs genes.

INTRODUCTION

Avian tibial dyschondroplasia (TD) is one of the most commonly occurring skeletal disorders, in which growth plate (GP) cartilage fails to develop into bone, causing lameness in chickens (Jahejo *et al*., 2021a). During bone development, chondrocytes become mature in response to bone marrow calcification in GP. After calcification, the chondrocytes multiply, grow and degenerate in bone marrow. Although studies have been done on TD, still it is the most challenging situation to treat because of the unknown etiology of the disease. However, suggested possible causes of TD include genetic predisposition, mal-nutrition, pesticide, and other chemical toxicity (Rath *et al*., 2007).

Thiram is well known for its cytotoxicity owing to its lipophilic nature, which enables it to bind with the cell membrane and exhibit its cytotoxic effects leading to disturbance in bone cartilage formation, bone

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0030-9923/2024/0002-0817 $ 9.00/0

degeneration, and immunosuppression (Beckmann *et al*., 2014; Jahejo *et al*., 2020). In our previous study, apoptosis and prostaglandins (PG) related genes have been identified in the erythrocytes of chickens affected with TD (Tian *et al*., 2013). As blood erythrocytes are a fundamental and substantial part of blood circulation, so the present study was designed to explore the variation in the expression of PRRs, cytokines, interferon, and *HDRs* genes in the chicken erythrocytes. Moreover, recent studies have shown the involvement of toll-like receptors (TLRs), *IL*, and cytokines in the immune response of chickens. Keeping in view these facts, we envisioned that *HDRs*, and *PRRs* genes could stimulate the immune functions in chicken erythrocytes under different disease conditions, including TD. In TD, immune response leads to enhanced phagocytosis resulting in the downregulation of interleukins and macrophages during bone development that is restored at normal levels after healing (Rath *et al*., 2005; Wang *et al*., 2018). Immune-related genes such as *HDRs*, and *PRRs*, interferon regulatory factor (*IRF*) 3, 5, 8, 10, and *TLR1*, 4, and 21 recognize various pathogens and are associated with molecular pathways in the innate and adaptive immune system of host defense (Belvin *et al*., 1996; Medzhitov *et al*., 2001). Immune-related genes play important role not only in the development of blood vessels but also in bone cartilage formation. Moreover, transcriptomes of HDRs, PRRs and certain cytokines were observed in chicken erythrocytes (Keiko *et al*., 2007; Paul *et al*., 2013). *HDRs* genes, including *IRF*3, 5, and 8, help host to fight against pathogens owing to their potent anti-microbial activity and ability to not only stimulate interferon system but also promote cell growth, immune response, phagocytic effect, RNA transcription, injury and bone healing (Keiko *et al*., 2007; Xiao *et al*., 2014).

Glutathione-S-transferase (GSTs) is the most important detoxifying enzyme family which is associated with catalyzing the conjugation of glutathione (GSH) into various exogenous and endogenous compounds such as thiram (Jahejo *et al*., 2021b; Danyelle *et al*., 2003). This previous study revealed the differential expression of rGSTA3 transcriptome in chondrocytes affected with thiram-induced TD (Tian *et al*., 2013). Recent findings also indicated that GSTs which play a major role in biosynthesis of PGs can further affect the differentiation of chondrocyte and matrix synthesis (Chang *et al*., 1987; Li *et al*., 2004; O'Keefe *et al*., 1992). However, *HDRs*, and *PRRs* genes (*IRF*3, 5, 8), *TLR*1, 4, and 21 have shown their extensive involvement in GST-based resistance in cytotoxic and genetic effects (Pena *et al*., 1997; Hilary *et al*., 2001). Rigid mass of nonviable cells have been produced in the tibial cartilage after the occurrence of TD, which suppressed the vessels. In-addition the level of erythrocytes, HCT (hematocrit) and Hb (hemoglobin) also reduce at the initial stage of TD (Defu *et al*., 2014; Huang *et al*., 2017). Furthermore, it has also been investigated that clinical signs in TD can be improved by GP vascularity and avoiding the accumulation of an excess of dead chondrocytes or disruption of normal chondrocyte differentiation that ultimately leads to overt locomotion problems (Street *et al*., 2002; Zhang *et al*., 2013). Therefore, these evidences will open new horizons for research to explore the exact cause of TD, which can be prevented and treated by influential genes associated with immune response in chicken erythrocytes and concerned cell survival/chondrocyte differentiation. However, the natural role of *HDRs*, and *PRRs* genes of avian erythrocyte in thiram-induce TD is not yet explored. Therefore, the main objective of the present study was to explore the effect of rGSTA3 protein through *HDRs*, and *PRRs* genes mRNA expression in chicken erythrocytes.

MATERIALS AND METHODS

Ethical approval

All the experimental procedures were approved by the College of Animal Science and Veterinary Medicine of Shanxi Agricultural University, China (Approval No. SXAU-EAW-2019Br.002003).

Experimental design

One hundred twenty arbor acres (AA+) broiler day-old chicks were purchased from Shanxi Daxiang Farming Group (Shanxi, China) and reared in battery brooders. Basal diet was prepared in accordance with the guidelines of the National Research Council (NRC, 1994). All chicks were provided *ad-libitum* basal diet and freshwater for the first week. On the 7th day, all the chicks were starved overnight and randomly allotted into six groups: A, B, C, D, E, and F (having 20 chicks in each group). Group A, B, and C was treated with 0, 20, 50 $\mu g \cdot kg^{-1}$ rGSTA3 protein, respectively, while groups D, E and F were treated with 100 $\mu g \cdot kg^{-1}$ thiram in addition to the supplementation of 0, 20, 50 $\mu g \cdot kg^{-1}$ of protein rGSTA3. To induce TD, 100 $mg \cdot kg^{-1}$ of thiram was given to groups D, E, and F for 48 h, as previously described by Tian *et al*. (2013). The thiram (JL131223002) was purchased from a commercial company (Waltham, MA, USA). The blood samples (2.5 mL) were collected from birds on 6th and 10th day as described previously by Niu *et al*. (2022). After completion of the trial, selected birds were sacrificed by cervical dislocation under euthanasia.

Blood sampling and erythrocyte extraction

Blood samples (2 mL each) from subcutaneous veins were collected and diluted with the same volume of Alsever's solution (Solarbio, Beijing, China). The diluted blood was added carefully into a 4 mL Histopaque-1119

solution (Sigma-Aldrich, USA) and centrifuged at 500 r/min for 20 min; subsequently the supernatant was removed as described previously (Niu *et al*., 2018). After that, erythrocytes were washed 4 times by mixing with phosphate buffer saline (PBS) at 400 r/min for 5 min and then centrifuged at 500 r/min for 20 min as described previously. After separation, erythrocytes were stained with Wright–Giemsa stain revealing greater than 99.9% purity.

RNA extraction and cDNA synthesis

Total RNA was extracted from erythrocytes using an RNAiso Plus kit (Takara Bio Inc., Dalian, China). The quantity of extracted RNA was determined by the Nano-Drop Bio analyzer ND1000 (Labtech, Uckfield, UK), and quality was checked by 1.5% agarose gel electrophoresis. The cDNA was prepared from total RNA by using the reverse transcription kit (Prime Script RT reagent Kit; Takara Bio Inc., China).

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

To analysis relative expression of *HDRs*, and *PRRs* genes qRT-PCR was performed using a qPCR kit (TaKaRa SYBR Premix Ex TaqTM II; Takara Bio Inc., China), using already reported primers synthesized by the Shanghai Generay Biotech Co., Ltd. (Shanghai, China). Details of primers and their respective annealing temperatures are presented in Table I. The relative expression of HDRs, PRRs, and their housekeeping gene (*18S rRNA*) were analyzed by Quant Studio™ 6 Flex Real-Time PCR System Software (Applied Biosystems, USA).

Statistical analysis

Data were expressed as mean and standard deviation (mean ± SD). A *P*-value < 0.05 was considered as statistically significant. Data were analysed using one-way analysis of variance (ANOVA) using JMP software. The student's t-test was performed to compare the significant differences between the means ($P < 0.05$).

RESULTS

TD index

Morphological characteristics of TD was measured by TD index, revealing quite different attributes in thiram group and rGSTA3 group. All thiram-treated birds showed quite higher (1.891 ± 0.08^{a}) TD index than rGSTA3 (0.09 ± 0.08^{b}) group, showing typical lesions in the proximal GP (Fig. 1).

Analytical effect of thiram in TD

Results revealed obvious effects of thiram-induced TD on day 6 when TD reached at a peak; after that, clinical signs started to diminish on d 10. The expression pattern of HDRs and PRRs transcripts at 6 and 10 d relative to the housekeeping gene *18S rRNA* was determined (Fig. 2).

Table I. The Primer sequences and accession numbers of immune-related genes used in quantitative RT- PCR.

Genes	Primary Sequence (5'→3')	Annealing Temperature	Gen Bank accession number
IRF3	**F:** CCAAGGAGTCCAAGCTCATC **R :** CGATAAGCTCGAAGAGGTTGA	56°C	NM_205372.1
IRF5	**F:** ACAGACCCAAGGAGAAGAAGCT **R:** TCCACCAGAGCATCCTTCAG	57°C	NM_001031587.1
IRF8	**F:** GGCTGATCGAGCAGATTGAC **R:** AAACAGCCCAAGCCTTGAAA	56°C	NM_205416.1
IRF10	**F:** CGGGATGCAGAGAAGGATGA **R:** CTCTGCTGGATGGGCAGTTA	57°C	XM_025142159.1
TLR1	**F:** GCGGCAAGTCCGAGCTAC **R**: AGCCAGGAAGCTGTACCA	55°C	NC_006091.5
TLR4	**F:** AGTCTGAAATTGCTGAGCTCAAAT **R:** GCGACGTTAAGCCATGGAAG	57°C	FJ_915520.1
TLR21	**F**: AAGGACCAGGAGGAGAAAT **R**: AGAGCCGAAATGAAGAACC	55°C	NM_001030558.1
MHC-II	**F:**ACGACATTGAGGCCGACCACGTAG **R:**TCTATAAACACCGTCTGCGACTGAC	56°C	CAJ_76977.1
18SrRNA	**F:** TTCCGATAACGAACGACAC **R:** GACATCTAAGGGCATCACAG	55°C	FM_165414

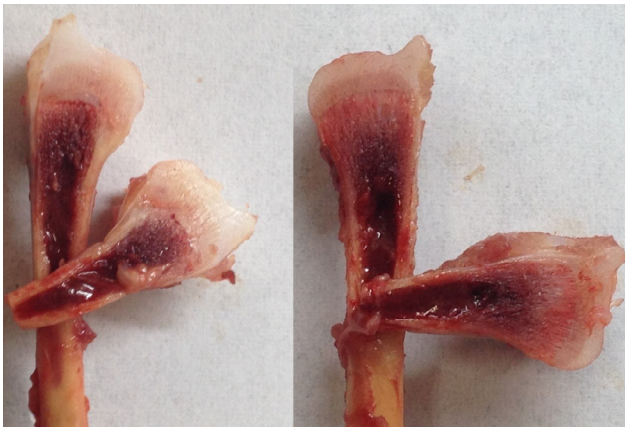

Fig. 1. Avian tibial bones with TD showed a significant difference between the control group and treatment group in TD index. Thiram fed induced tibial bones on (left) and control on (right). However, a tibial bone induced by thiram is supplemented, showing TD on the top of proximal growth plate.

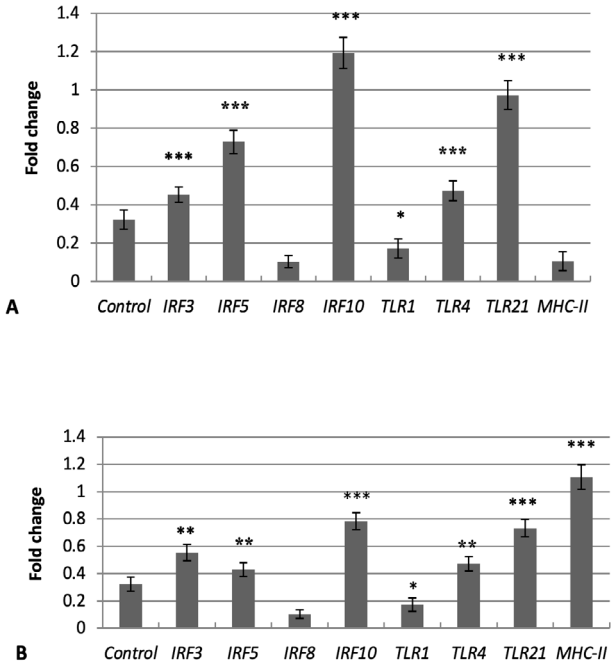

Fig. 2. The expression pattern of TLRs and immune related genes *TLR1*, 4, 21, *IRF3*, 5, 8, 10, and *MHC-II* in chicken erythrocyte, induced with thiram on day 6 (A) and day 10 (B). Expression levels of *TLRs* and immune-related genes were relatively considered to that of *18S rRNA* the housekeeping genes via quantitative real-time PCR, IRF10 mRNA in A, *MHC-II* mRNAs in B was highly significant up-regulated along with P values which were less than 0.05 and SEM (standard error of mean) represented by error bars.

Biological expression of erythrocytes at the transcript level in HDRs and PRRs

The TLR family is an extremely conserved group of proteins that contribute to innate, adaptive immune response initiation, regulation, and recognition of pathogens. The primary purpose of innate immunity is to activate the host immune signalling pathways, which is mediated PRRs. The relative expression of *HDRs* and *PRRs* in chicken erythrocytes was determined by measuring their transcript levels in treatment and control groups by qRT-PCR (Fig. 2). This study revealed that chicken erythrocyte substantially expressed transcript levels for interferon regulatory factor (*IRF*) of 3, 5, 8, and 10, *TLR*1, 4, 21, and *MHC-II*. However, among the transcripts *IRF*10 was the most dominant transcript in *HDRs* and *PRRs* , while, *IRF*8 was the least frequent among the control and experiment groups.

Consequences of protein rGSTA3 and thiram-induced TD on the mRNA expression of HDRs *and* PRRs *genes in chicken erythrocytes.*

Variable effect of rGSTA3 protein in thiram-induce TD was observed on the expression of *HDRs* and *PRRs* genes in erythrocytes on days 6 and 10 (Fig. 3). On day 6, in the group which was given 50 $\mu g \cdot kg^{-1}$ rGSTA3 protein resulted significant up-regulation of *HDRs* and *PRRs* compared with control group ($P < 0.05$). The transcripts expression of *IRF3*, 5, 8, and 10 genes in thiram treated groups were different than control groups, revealing significant up-regulation of mRNA expressions with stimulation of 50 $\mu g \cdot kg^{-1}$ rGSTA3 protein in thiram treated chickens ($P < 0.05$). Meanwhile, the mRNA expression of *IRF10* was up-regulated ($P < 0.05$) in thiram treated groups. However, the mRNA expression of *IRF8*, *TLR1*, and *MHC-II* was significantly reduced ($P < 0.05$) on day 6 in rGSTA3 group. On day 10, the expression of *IRF3* and *TLR1* was reduced. Furthermore, *IRF*3, 5, 10, *TLR*4, 21 and *MHC-II* comparatively dose dependent by the stimulation of protein rGSTA3 in the experimental group in which thiram was used as feed additive. In addition after the 50 $\mu g \cdot kg^{-1}$ rGSTA3 protein stimulation the highest up-regulation was deducted for *IRF*10 as compared to the control group ($P < 0.01$).

DISCUSSION

The TD is an avian pervasive disease and the most emerging problem in fast-growing chicken strains that result in severe leg problems due to abnormal development of bone tissue. The current study provided evidence that this condition is a manifestation of impairment of vascularization, which negatively affects the availability

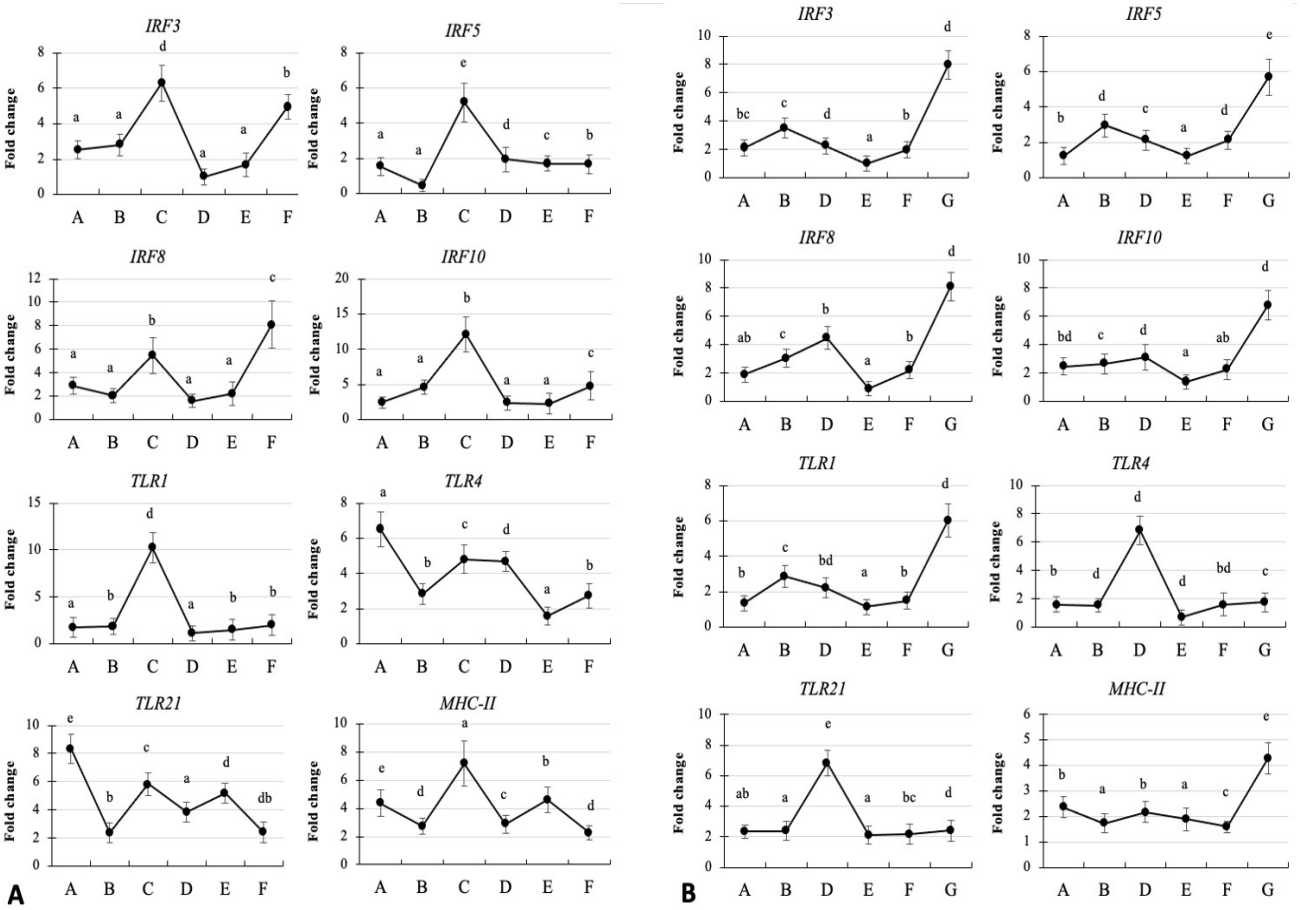

Fig. 3. The mRNAs expression pattern of *TLR*s and immune-related genes *TLR*1, 4, 21, *IRF*3, 5, 8, 10, and and *MHC-II* in chicken erythrocyte, induced with thiram on day 6 (A) and day 10 (B). Basal diet containing groups (A, B and C) treated through 0, 20, 50 $\mu g \cdot kg^{-1}$ of recombinant glutathione S transferase A3 (rGSTA3) protein, and thiram-containing diet groups (D, E and F) treated through 0, 20, 50 $\mu g \cdot kg^{-1}$ of rGSTA3 protein, respectively. In each chick groups, different lowercase lettering (a–e) point out statistically significant differences ($P < 0.05$) and SEM (standard error of mean) represented by error bars.

and distribution of minerals and nutrients. Minerals like calcium and phosphorus are crucial for bone formation, which is a complex process being affected by a number of factors such as genetics, malnutrition, toxins, and diseases. In abnormal bone growth, erythrocytes are most active cells that stimulate biological process which directly affect defense mechanism responsible for immune response in the form of cytokines, interleukin (*IL*), *TLRs* and *IRFs* to fight against microorganisms and alleviate adverse effects of toxins (Morera and MacKenzie, 2011; Ricardo *et al*., 2011; 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). Moreover, nutrients are supplied to chondrocytes through blood vessels mainly by the accumulation of tibial bone erythrocytes during normal bone growth (Huang *et al*., 2017). These erythrocytes are also responsible for generating an immune-response in chickens to fight against disease and toxic compounds. These evidences show that the immune response of chicken erythrocyte during vascularization of bone is strongly associated with TD (Orth *et al*., 1994; Krause *et al*., 2003; Paolucci *et al*., 2013).

Present study, evaluated that the immune-related genes (*HDRs* and *PRPs*) that actively participate in preventing the diseases through stimulating the innate and adaptive immune system with subsequent positive effects on TD index. The findings revealed quite a higher TD index in thiram treated group exhibiting characteristic lesions in proximal GP as compared to rGSTA3 (Rath *et al*., 2007; Nabi *et al*., 2018). Even in the rGSTA3 protein receiving group, TD index was lower, but it produced

enormous changes in thiram induce TD, indicating a positive effect on the expression of *HDRs* and *PRPs* genes in erythrocytes. These findings indicate that transcription of *HDRs* and *PRPs* genes was constitutively up-regulated with stimulation of rGSTA3 protein in the erythrocytes of thiram-induced TD. Among *HDRs* and *PRPs*, the mRNA expression of *IRF*3, 5, 8, 10 *TLR*1, 4, 21, and *MHC-II* was quite different at 6 and 10 d chicks, as it was undulating at 6 d it was while at 10 d such genes expressed widely in which transcripts of *MHC-II* were most dominant in chicken erythrocytes. Similar findings regarding wider expression of *IRF*s, *TLR*s, and *MHC-II* in chicken neural cells like glial cells, skin, heart and blood of human beings have been reported earlier (Keiko *et al*., 2007; Gay *et al*., 2007; Zhang *et al*., 2014; Han *et al*., 2018). However, results of the present study revealed that *IRF*10, *TLR*21 and *MHC-II* mainly play important role in different tissues leading to enhanced immune response that provide protection against many diseases in chicken including TD. Surprisingly, among *IRF*s some genes like *IRF10* showed quite higher mRNA expression in thiram-induced TD. Whereas, the expression of *TLR1*, and *MHC*-II was decreased at 6 d. However, at d 10, quite contrasting result was observed in case of *MHC-II* as its expression broadly induced by thiram which poses a negative effect on tissues development during cartilage formation, while the expression of *IRF*-3, 5, 10 and *TLR*21 was higher as compared to its level at d 10. Moreover, chicks at 6 d exhibited a significant decrease in *IRF*-3, 5, and *TLR*21 transcription in thiram-induced TD as compared to 10 d. Moreover, reduction in the transcription of *IRF*8, and *TLR*1 at 6 and 10 d was observed and *MHC-II* in 6 d, but the level of *IRF*10, and *MHC-II* were quite higher in these birds. These findings proved that thiram-induced TD could affect immune response mediated by various *HDRs* and *PRPs* genes, which caused discontinuity at certain extent and disturbed the immune response of chicken.

Immune-related genes interfere with antiapoptotic action of proteins which is used as TD recovery agent that suppressed erythrocytes and reduced the blood circulation at initial stage of TD (Li *et al*., 2004; Lynn, 2003; Wang *et al*., 2018). This evidence indicated that thiram-induce TD negatively affected the blood origin pathways leading to disturbance of blood, mineral and nutrient exchange in the proximal region of GP, which subsequently resulted in death of chondrocytes (Marikovsky, 2002; Rath *et al*., 2007). Dead chondrocytes are the key lesion of TD caused by the suppression of immune-related genes. For the first time, we report *TLR*4 suppression in infected chicken erythrocytes. Moreover, this study also evaluated the genes related to TLR pathway revealing the negative effect of *TLR*4 suppression on chondrocytes, leading to their subsequent death. These findings agree with previous studies reporting suppression of *TLR*2 mRNA expression in different cells and tissues of chicken such as erythrocytes may be associated with different diseases including TD.

The present study showed the transcripts of *HDRs* and *PRR*s genes (*IRF-3, 5*, and *MHC-II*) were significantly suppressed at 6 d in broilers but exhibited significant up-regulation at 10 d with the stimulation of 50 $\mu g \cdot kg^{-1}$ rGSTA3 protein. These findings envisaged that rGSTA3 protein could play vital role in stimulating *HDRs* and *PRPs* gene expression and induction of IRFs and *TLR*s which might stimulate immune response at initial and later stages of TD. However, higher rGSTA3 level brings a normal point in the later or recovery phase of TD. The expression of *IRF*3, 5, coupled with *MHC-II* was up-regulated with the stimulation of 50 $\mu g \cdot kg^{-1}$ rGSTA3 proteins. The result showed that *IRF*10, *TLR*21 and *MHC-II* 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 *MHC-II* in erythrocytes. The findings of current study suggested that rGSTA3 protein could stimulate an enormous immune response in the body by initiating the expression of various *HDR*s and *PRR*s genes in chicken erythrocytes.

Moreover, findings of current study concluded that rGSTA3 protein plays vital role in the recovery of TD in chicken through determining the protein expression of antigen-processing gene *MHC*-II molecules in chicken erythrocytes, their over expression in osteoblasts, main bone cells could have initiated inflammatory immune responses in various bone diseases including TD (Schrum *et al*., 2003; Nagahama *et al*., 2004). Furthermore, *IRF8* a prominent member of *HDRs* genes, is generally considered as a promoter that up-regulates the expression of *IFN* type-I and II, and *IL* which subsequently stimulates B cells and $CD8^+$ T cells that kill microbes and enhance tissue implantation. Moreover, such expression is closely associated with MHC-II molecules (Hilchie *et al*., 2013; Xiao *et al*., 2014). Moreover, *IRF* family members act as a constitutive immunomodulator which customize the reaction of pre and post inflammation and anti-inflammatory reaction through regulating vascular injury, mediation of immune system during wound-healing and apoptosis (Xiao *et al*., 2014).

The present study showed up-regulation of *IRF*10 with a maximum fold expression as a result of supplementation with 50 $\mu g \cdot kg^{-1}$ of rGSTA3 protein between treatment and control group. These findings corresponded well with *IRF*10, and *MHC-II* transcripts which showed highest expression in chicken erythrocytes in thiram-induce TD. Therefore, these findings open a new horizon for research

to explore the potential of rGSTA3 to stimulate the *IRF*10 expression in chicken erythrocyte to regulate GP vascularity and chondrocyte maturation. However, further studies are required to corroborate these findings that how HDRs genes can stimulate blood erythrocytes to enhance their biological functions during the development of TD. Finally, the results collectively revealed that *HDRs* and *PRRs* genes perform potential immune modulatory role in chicken erythrocytes during thiram-induce TD. Immune-related genes provide defense mechanism through TLRs pathway, which distinguishes a small number of proinflammatory factors produce during TD. Moreover, these also mediate JAK/STAT pathways that promote inducible intracellular signals to induce *HDRs* and *PRPs* genes along with expression of proinflammatory cytokines that collectively promote cell growth and phagocytic activity (Keiko *et al*., 2007).

CONCLUSION

The present study concluded that *HDR* and *PRP* genes (*IRF3*, 5, 8, 10, *TLR1*, 4, 21, and *MHC*-II) play an important role in host immunity during thiram-induce TD in broilers. The expression of these genes showed wide variation in blood erythrocytes as genes which were down-regulated in thiram treatment were become up-regulated by stimulation with rGSTA3 protein. Collectively, the recent finding revealed that expression of selected *HDR* and *PRP* genes (*IRF3*, 5, 8, 10, *TLR1*, 4, 21, and *MHC*-II) was associated with TD while rGSTA3 protein alleviated the adverse effects of TD through promoting the expressions of the selected gene. However, further molecular studies are required to corroborate these findings and elucidate the clear, dynamic mechanism in thiram-induced TD and its developmental stages.

ACKNOWLEDGEMENTS

The work was supported by the earmarked fund for Shanxi Agriculture Research System, Research Project Supported by Shanxi Scholarship Council of China (2017-073), the National Natural Science Foundation of China (31072179), Key Technologies R & D Program of Shanxi Province (20130311027-3), “131” Leading Talent Project for Colleges and Universities of Shanxi Province, the Fund for Shanxi “1331 Project” (20211331-3), the special fund for Science and Technology Innovation Teams of Shanxi Province and Shanxi Key Laboratory of Protein Structure Determination (202104010910006).

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Beckmann, R., Houben, A., Tohidnezhad, M., Kweider, N., Fragouilis, A., Wruck, C., Brandenburg, L., Hermanns, S.B., Goldring, M. and Pufe, T., 2014. Mechanical forces induce changes in VEGF-1/ sFlt-1 expression in human chondrocytes. *Int. J. mol. Sci*., **15**: 15456-15474. https://doi.org/10.3390/ijms150915456

Belvin, M.P. and Anderson, K.V., 1996. A conserved signaling pathway: The *Drosophila* toll-dorsal pathway. *Annu. Rev. Cell. Dev. Biol.*, **12**: 393–416. https://doi.org/10.1146/annurev.cellbio.12.1.393

Chang, M., Hong, Y., Burgess, J.R., Tu, C.P. and Reddy, C.C., 1987. II Isozyme specificity of rat liver glutathione-S-transferases in the formation of PGF2α and PGE2 from PGH2. *Arch. Biochem. Biophys.*, **259**: 548-557. https://doi.org/10.1016/0003-9861(87)90521-2

Danyelle, M.T. and Kenneth, D.T., 2003. The role of glutathione-S-transferase in anti-cancer drug resistance. *Oncogene,* **22**: 7369-7375. https://doi.org/10.1038/sj.onc.1206940

Defu, Y., Zong, S.Y., Changma, F and Runze, Y., 2014. Impact on red blood cell immunity patterns in postoperative phase following total hip arthroplasty. *Cent. Eur. J. Immunol*., **39**: 377–383. https://doi.org/10.5114/ceji.2014.45951

Gay, C., Gilman, V. and Leach, Jr. R., 2007. Immuno-localization of vascularization factors in normal, tibial dyschondroplasia and rachitic cartilage. *Avian Pathol.,* **36**: 445-451. https://doi.org/10.1080/03079450701591387

Han, P., Wang, S., Zhang, Q., Zhang, S., Shao, R., Xu, W., Zhang, W., Xu, Q., Wei, Q. and Qi, Z., 2018. Molecular characterization and expression analysis of TLR1 and TLR4 from the endangered fish Dabry’s sturgeon (*Acipenser dabryanus*). *Dev. Comp. Immunol*., **86**: 180e188. https://doi.org/10.1016/j.dci.2018.05.009

Hilary, R., Louise R., Federica, O., Betty, J., Xuelan, W., Charles W. Roth., Frank H. Collins and Janet H., 2001. Identification of a novel class of insect glutathione-S-transferases involved in resistance to DDT in the malaria vector Anopheles gambiae. *Biochem. J.*, **359**: 295-304. https://doi.org/10.1042/0264-6021:3590295

Hilchie, A.L., Wuerth, K., and Hancock, R.E., 2013. Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. *Nat. chem. Biol*., **9**: 761-768. https://doi.org/10.1038/nchembio.1393

Huang, S., Rehman, M., Lan, Y., Qiu, G., Zhang, H.,

Iqbal, M., Luo, H., Mehmood, K., Zhang, L. and Li, J., 2017. Tibial dyschondroplasia is highly associated with suppression of tibial angiogenesis through regulating the HIF-1A/VEGF/VEGFR signaling pathway in chickens. *Sci. Rep*., **7**: 9089. https://doi.org/10.1038/s41598-017-09664-6

Jahejo, A.R. and Tian W.X., 2021a. Cellular, molecular and genetical overview of avian tibial dyschondroplasia. *Res. Vet. Sci.,* **135**: 569-579. https://doi.org/10.1016/j.rvsc.2020.10.002

Jahejo, A.R, Jia F.J., Raza S.H., Shah M.A., Yin J.J., Ahsan A., Waqas M., Niu S., Ning G.B., Zhang D. and Khan A., 2021b. Screening of toll-like receptor signaling pathway-related genes and the response of recombinant glutathione-S-transferase A3 protein to thiram induced apoptosis in chicken erythrocytes. *Dev. Comp. Immunol.,* **114**: 103831. https://doi.org/10.1016/j.dci.2020.103831

Jahejo, A.R., Zhang, D., Niu, S., Mangi, R.A., Khan, A., Qadir, M.F., Khan, A., Chen, H.C. and Tian, W.X., 2020. Transcriptome-based screening of intracellular pathways and angiogenesis related genes at different stages of thiram induce tibial lesion in broiler chickens. *MBC Genom.,* **12864**: 020-6456-9. https://doi.org/10.1186/s12864-020-6456-9

Keiko, O., Prafullakumar, T. and Toru K., 2007. The interferon regulatory factor family in host defense mechanism of action. *J. biol. Chem*., **282**: 20065–20069. https://doi.org/10.1074/jbc.R700003200

Krause, A., Sillard, R., Kleemeier, B., Klüver, E., Maronde, E., Conejo-García, J.R. and Wattler F., 2003. Isolation and biochemical characterization of LEAP-2, a novel blood peptide expressed in the liver. *Prot. Sci.,* **12**: 143-152. https://doi.org/10.1110/ps.0213603

Lee, Y.J., Lee, J.S., Kang, E.H., Lee, Y.K., Kim, S.Y., Song, Y.W. and Koo, K.H., 2012. Vascular endothelial growth factor polymorphisms in patients with steroid-induced femoral head osteonecrosis. *J. Orthop. Res.,* **30**: 21-27. https://doi.org/10.1002/jor.21492

Li, T.F., Zuscik, M.J., Ionescu, A.M., Zhang, X., Rosier, R.N., Schwarz, E.M., Drissi, H. and O'keefe, R.J., 2004. PGE2 inhibits chondrocyte differentiation through PKA and PKC signaling. *Exp. Cell Res*., **300**: 159-169. https://doi.org/10.1016/j.yexcr.2004.06.019

Lynn, D.J., Lloyd, A.T. and O'Farrelly, C., 2003. In silico identification of components of the Toll-like receptor (TLR) signaling pathway in clustered chicken expressed sequence tags (ESTs). *Vet. Immunol. Immuno. Pathol.,* **93**: 177-184. https://doi.org/10.1016/S0165-2427(03)00058-8

Marikovsky, M., 2002. Thiram inhibits angiogenesis and slows the development of experimental tumours in mice. *Br. J. Cancer,* **86**: 779-787. https://doi.org/10.1038/sj.bjc.6600078

Medzhitov, R., 2001. Toll-like receptors and innate immunity. *Nat. Rev. Immunol.*, **1**: 135–145. https://doi.org/10.1038/35100529

Morera, D. and MacKenzie, S.A., 2011. Is there a direct role for erythrocytes in the immune response? *Vet. Res*., **42**: 89. https://doi.org/10.1186/1297-9716-42-89

Nabi, F., Iqbal, M.K., Zhang, H., Rehman, M.U., Shahzad, M., Huang, S., Han, Z., Mehmood, K., Ahmed, N., Chachar, B., Arain, M.A. and Li, J., 2018. Clinical efficiency and safety of Hsp90 inhibitor Novobiocin in avian tibial dyschondroplasia. *J. Vet. Phar. Ther.,* **41**: 902-911. https://doi.org/10.1111/jvp.12692

Nagahama, K., Aoki, K., Nonaka, K., Saito, H., Takahashi, M., Varghese, B. and Ohyama, K., 2004. The deficiency of immunoregulatory receptor PD-1 causes mild osteopetrosis. *Bone,* **35**: 1059-1068. https://doi.org/10.1016/j.bone.2004.06.018

Niu, S., Jahejo, A.R., Jia, F.J., Li, X., Ning, G.B., Zhang, D., Ma, H.L., Hao, W.f., Gao, W.W., Zhao, Y.J., Gao, S.M., Li, G.L., Li, J.H., Yan, F., Gao, R.K., Bi, Y.H., Han, L.X., Gao, G.F. and Tian, W.X., 2018. Transcripts of antibacterial peptides in chicken erythrocytes infected with Marek's disease virus. *BMC Vet. Res.,* **14**: 363. https://doi.org/10.1186/s12917-018-1678-7

Niu, S., Jahejo, A.R., Jia, F.J., Li, X., Ning, G.B., Zhang, D., Ma, H.L., Hao, W.f., Gao, W.W., Zhao, Y.J., Gao, S.M., Li, J.H., Li, G.L., Yan, F., Gao, R.K., Chen, H.C. and Tian, W.X., 2019. 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. *Pakistan J. Zool.,* **51**: 1475-1482. http://dx.doi.org/10.17582/journal.pjz/2019.51.4.1475.1482

NRC, 1994. Nutrient requirements of poultry: 1994. National Academies Press.

O'Keefe, R.J., Crabb, I.D., Puzas, J.E. and Rosier, R.N., 1992. Influence of prostaglandins on DNA and matrix synthesis in growth plate chondrocytes. *J. Bone Min. Res.,* **7**: 397-404. https://doi.org/10.1002/jbmr.5650070407

Orth, M. and Cook, M., 1994. Avian tibial dyschondroplasia: A morphological and

biochemical review of the growth plate lesion and its causes. *Vet. Pathol.,* **31**: 403-414. https://doi.org/10.1177/030098589403100401

Paul, S.T., M., Paolucci, S., Barjesteh, N., Wood, R.D. and Sharif, S., 2013. Chicken erythrocytes respond to toll-like receptor ligands by up-regulating cytokine transcripts. *Res. Vet. Sci.*, **95**: 87-91. https://doi.org/10.1016/j.rvsc.2013.01.024

Paolucci, F.C., Tamburini, M.V., Ambretti, S. and Lazzoarotto, T., 2013. Comparison between MALDI-TOF MS and film array blood culture identification panel for rapid identification from blood culture. *J. Microbiol. Meth.,* **104**: 92-93. https://doi.org/10.1016/j.mimet.2014.06.018

Peña, S.V. and Krensky, A.M., 1997. Granulysin, a new human cytolytic granule-associated protein with possible involvement in cell-mediated cytotoxicity. *Semin. Immunol*., **2**: 117-125. https://doi.org/10.1006/smim.1997.0061

Rath, N.C., Huff, W.E. and Huff, G.R., 2005. Effect of humic acid on broiler chickens. *Poult. Sci*., **86**: 2390-2395. https://doi.org/10.3382/ps.2007-00219

Rath, N.C., Kannan, L., Pillai, P.B., Huff, W.E., Huff, G.R., Horst, R.L. and Emmert, J.L., 2007. Evaluation of the efficacy of vitamin D3 or its metabolites on thiram-induced tibial dyschondroplasia in chickens. *Res. Vet. Sci*., **83**: 244–250. https://doi.org/10.1016/j.rvsc.2006.12.008

Ricardo, F.A., Cla´udia, B., Margarida M. and Fernando, A.A., 2011. Red blood cells release factors with growth and survival bioactivities for normal and leukemic T cells. *Immun. Cell. Biol*., **89**: 111–121. https://doi.org/10.1038/icb.2010.60

Schrum, L.W., Bost, K.L., Hudson, M.C. and Marriott, I., 2003. Bacterial infections induce expression of functional MHC- class-II molecules in murine and human osteoblasts. *Bone,* **33**: 812-821. https://doi.org/10.1016/S8756-3282(03)00265-5

Street, J., Bao, M., Bunting, S., Peale, F.V., Ferrara, N.,Steinmetz, H., Hoeffel, J., Cleland, J.L., Daugherty, A., van Bruggen, N. and Redmond, H.P., 2002. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc. natl. Acad. Sci. U.S.A.,* **99**: 9656-9661. https://doi.org/10.1073/pnas.152324099

Tian, W.X., Li, J.K., Qin, P., Wang, R., Ning, G.B., Qiao, J.G., Li, H.Q., Bi, D.R., Pan, S.Y. and Guo, D.Z., 2013. Screening of differentially expressed genes in the growth plate of broiler chickens with tibial dyschondroplasia by microarray analysis. *BMC Genom.*, **14**: 276. 1471- 2164-14-276. https://doi.org/10.1186/1471-2164-14-276

Wang, C.X., Niu, S., Jahejo, A.R., Jia, F.J., Li, Z., Zhang, N., Ning, G.B., Zhang, D., Li, H.Q., Ma, H.L., Hao, W.f., Gao, W.W., Gao, S.M., Li, J.H., Li, G.L., Yan, F., Gao, R.K., Zhao, Y.J., Chen, H.C. and Tian, W.X., 2018. Identification of apoptosis-related genes in erythrocytes of broiler chickens and their response to thiram-induced tibial dyschondroplasia and recombinant glutathione-S-transferase A3 protein. *Res. Vet. Sci*., **120**: 11-16. https://doi.org/10.1016/j.rvsc.2018.08.001

Zhang, X.J., Jiang, D.S. and Li H., 2014. The interferon regulatory factors as novel potential targets in the treatment of cardiovascular diseases. *Br. J. Pharm*., **10**: 1111. https://doi.org/10.1111/bph.12881

Zhang, J.P., Deng, Y.F., Zhou, Z.L. and Hou, J.F., 2013. Expression and identification of recombinant chicken vascular endothelial growth factor in *Pichia pastoris* and its role in the pathogenesis of tibial dyschondroplasia. *Poult. Sci.,* **92**: 3214-3227. https://doi.org/10.3382/ps.2013-03420