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RESEARCH ARTICLE

Ameliorative Effects of Triptolide against Autophagy and Apoptosis in Thiram Induced Tibial Dyschondroplasia

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ABSTRACT

Tibial dyschondroplasia (TD) is a metabolic bone disease that occurs in fast-growing chickens and can lead to substantial economic damages and compromise the poultry welfare. Triptolide is a traditional Chinese medicine (TCM), which has been broadly used as an anti-inflammatory, and anti-cancerous drug. However, its therapeutic role against tibial dyschondroplasia is under-reported yet. Therefore, the study aimed for investigating the protective effect of triptolide against autophagy and apoptosis in TD affected chickens. Random classification of chickens (n=30) was done into three groups: CON, TD and triptolide treatment group (TP). All chickens were given standard diet until the end of the experiment for 18 days. In this study, tibial bone was collected for analyzing several aspects, serum was collected for biochemical analysis, and microscopic assessment was done by H&E staining. Immunohistochemistry, immunofluorescence, western blotting, and RT-qPCR were used to detect autophagy and apoptosis-related proteins and genes. Observations illustrated that after triptolide treatment, the symptoms of TD group were improved, and tibial parameters were changed. At the same time, the expression of m-TOR and P62 mRNA in TP group showed significant decrease comparing to TD group. Precisely, thiram induced TD was found to be involved in hypertrophic chondrocytes apoptosis and autophagy, and triptolide showed protective effects against TD.

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INTRODUCTION

Thiram (tetramethylthiuram disulfide) is a fungicide which is widely used in agriculture. It has high efficiency and low toxicity characteristics, but it also causes harm to the environment and living body. It is responsible for stunt growth and bone deformation in broilers, so it is widely used to induce tibial dyschondroplasia (TD) (Liu *et al.,* 2022).

TD is a common deformity of the leg bones in fast-growing poultry, that cause huge financial losses to poultry industry. Poultry with TD suffer from depression and reduced diet intake, leading to a decreased growth performance (Mehmood *et al.,* 2018). At the same time, affected chickens also present difficulties in standing, movement disorders and wing support walking. Growth plate (GP), which is located between the epiphysis and the diaphysis, forming a multilayered structure in the shape of a sandwich. Chondrocytes in growth plate can be found in four areas: 1) Resting zone, it contains pairs of small, uniform round chondrocytes; 2) Proliferative zone, chondrocytes have strong division activity and are differentiated into flat cells to form vertical columns, namely cell columns; 3) Prehypertrophic zone, chondrocytes lack cell division. At this point, hypertrophic chondrocytes constitute hypertrophic areas; 4) Hypertrophic zone, here hypertrophic chondrocytes are replaced by calcified matrix and invading blood vessels are found (Burdan *et al.,* 2009).

Characteristics of TD are non-vascularised, mineralized-free, and without calcification cartilage in

chicken tibial growth plates (TGPs), where endochondral bone formation fails. Several studies have suggested that the occurrence of TD is related to abnormal generation of blood vessels (Jahejo and Tian 2021). TD disrupts the proliferation of chondrocytes in the GPs during long bone development and prevents them from stacking properly together to promote a columnar layer of cells formation. As a result, chondrocytes fail to differentiate into hypertrophic chondrocytes, leading to apoptosis of chondrocytes. Chondrocytes accumulate in hypertrophic areas where blood vessels cannot invade, eventually forming an enlarged, unmineralized and non-vascularized growth plate (Li *et al.*, 2020).

Apoptosis is a development by which the cells stop growing and dividing, is sometimes called programmed cell death. Bcl-2 proteins involve in the apoptotic pathway and act as cellular-damage signaling, regulating a unique apoptotic signal to initiate the permeability of mitochondrial outer membrane. p53 as one of the most important tumor suppressor genes, can engage the expression of different multiple pro-apoptotic gene products and can show its anti-cancer effect by activating cell death, as well as cell cycle arrest in cancer cells (D'Arcy 2019; Wang *et al.*, 2021). Autophagy is an important cellular mechanism that maintains body homeostasis. The process of autophagy is tightly controlled by different regulatory components from start to finish. The abnormal regulation of m-TOR makes the differences in the pathophysiological processes of cancer, diabetes, aging, cardiovascular diseases. The Beclin1 protein acts as the mammalian orthologue of the yeast autophagy protein Atg6, which play an important role in the development of autophagy. The interaction between the Beclin1 and the Bcl-2 leads to the interconversion of autophagy and apoptosis. The accumulation of P62 enhance, which directly interacts with LC3 to be incorporated subsequently into the autophagosome (Ma *et al.*, 2018; Yi *et al.*, 2022).

Studies have demonstrated that autophagy and apoptosis play crucial role in bone homeostasis. Tumor necrosis factor (TNF-α) contributes to the induction of autophagy in osteoblasts, and enhance autophagy to protect cells by reducing TNF-α-induced apoptosis (Zheng *et al.*, 2017). In vitro and in vivo, autophagy induces osteoclast differentiation and promotes bone resorption mediated by osteoclast. The number of apoptotic osteoblasts and osteocytes accumulates during aging, eventually reducing osteoblast number and bone formation.

Triptolide is isolated from a Chinese herb, it is synonymous to PG490 or LLDT-2 (C20H24O6, molecular weight: 360.4). Triptolide is well known for its therapeutic role in RA, relieving mice with arthritic symptoms (Cheng *et al.*, 2021). Triptolide attenuates lipopolysaccharide (LPS)-induced NF-κB activity in astrocytes, microglia, or endothelial cells (Song *et al.*, 2019). It can prevent skeletal muscle atrophy by regulating protein synthesis/degradation pathways and inflammatory pathways (Fang *et al.*, 2021). As an autophagy regulator, it can also play neuroprotective role, and shows antitumor activity. It also helps in organ protection from toxicity and podocyte protection by regulating autophagy. This research aims to study the protective effect of *Tripterygium wilfordii* on thiram induced-TD chickens to regulate mitochondrial apoptosis and autophagy.

MATERIALS AND METHODS

Chicken management and experimental design: After three days of feeding, 30 one-day-old healthy white-feathered broilers were divided into CON, TD and TP. The diets of TD group and TP group were continually supplemented with 50mg/kg thiram for 2 weeks. At 7 days of age, the broilers of TP group were fed with 0.2mg/kg of triptolide (Shanghai yuanye Bio-Technology) dissolving in PBS containing DMSO by gavage according to their body weight, and other groups were fed with PBS solution containing the same amount of DMSO according to their body weight. Other conditions were consistent throughout the experiment which lasted for 18 days. The treatment was approved by the Animal Welfare and Research Ethics Committee of SCAU.

Tibial parameters analysis and sample collection: At 18 days of age, chickens were sacrificed by cervical dislocation and collected the blood samples for biochemical analysis. After that, measure the weight of chickens and the tibia bones, the width and length of the tibia bone and the size of TGPs. After dissection, fixed the tibial bones in paraformaldehyde (4%), and others were stored at -80°C.

Analysis of serum biochemical indices: The serum biochemical indices of albumin (ALB), lactate dehydrogenase (LDH), calcium (Ca), total bilirubin (T-Bil), direct bilirubin (D-Bil), carbamide (UREA), alkaline phosphatase (ALP), glutamic oxaloacetic transaminase (AST), phosphorus (P), glutamic pyruvic transaminase (ALT) and creatinine (CREA) were determined by a biochemical analyzer.

Histopathological analysis: The cartilage growth plates were processed using routine histological procedures on the basis of our previous studies (Li *et al.*, 2021) and examined under a microscope (Leica, Germany).

Immunohistochemistry and immunofluorescence analysis: For immunohistochemistry, the experimental steps were referred to prior research (Yang *et al.*, 2021). The Beclin1 primary antibodies (No. A11761, ABclonal Technology Co.,Ltd., Wuhan, China) were diluted with 1% BSA (1:1000).

Experimental steps for immunofluorescence were referred to prior research (Zhu *et al.*, 2022). Sections were incubated with the Beclin1 (No.A11761) and CASP3 (NO.A0214) primary antibody (ABclonal Technology Co., Ltd., Wuhan, China) which were diluted with 1% BSA (1:1000) overnight and secondary antibody (Cowin Biotech Co., Ltd., Jiangsu, China) for 1 h at 4°C. Finally, the cells were incubated in DAPI (Beyotime, China). Slides were secured and immediately examined under a fluorescence microscope (Leica; DM4, Germany).

RNA extraction and RT-qPCR analysis: The target genes primers were designed from the website of the NCBI (Table 1). Chicken cartilage mRNA was extracted with commercially available kit (Vazyme Biotech Co., LTD.). After reversing transcript into cDNA, ampilificat

Table 1: Primers for qRT-PCR.

Genes name	Forward sequence (3′→5′)	Reverse sequence (5′→3′)
Caspase-8	GAGCTGGCGTCTCTGAAGTT	CTTCCCGCTTCCTTACAGTG
Caspase-9	GCCAGATGCTGTCCCCTATC	CATTGGCAACCTGGGAAGGTG
LC3-B	AGTGAAGTGTAGCAGGATGA	AAGCCTTGTGAACGAGAT
Bcl-2	GATCGTCGCCTTCTTCGAGT	GGCCTCATACTGTTGCCGTA
Beclin 1	CGTATGGCAACCACTCGTATT	TTATTGTCCCAGAAGAACCTCAG
Bax	TCCTCATCGCCATGCTCAT	CCTTGGTCTGGAAGCAGAAGA
P62	GACCCAGCCAAGACTACCAT	CAGAGGCATGTAGTTTCGGC
Parkin	GTCCAGCAAAGCATCGTTCA	CAACGATGGAAGGATGCTGG
P53	GAGATGCTGAAGGAGATCAATGAGCACACATCCTGTTGCTCGTC	GTGGTCAGTCCGAGCCTTTT
APAF-1		TCCATTGTGCCTCAGGGTTT
Mtor	GGTGATGACCTTGCCAAACT	CCAACCATTGACATCACAGC
GAPDH	GTCGGTGTGAACGGATTTG	CAATCTCCACTTTGCCACTG

cDNA by qPCR. Repeated each experiment for 3 times. Analyzed the changes of mRNA levels of each gene via $2^{-\triangle\triangle CT}$ method and then normalized to the level of housekeeping gene GADPH.

Western blotting analysis: Each PVDF membrane was incubated with the CASP3(NO. A0214), CYTC (NO. A13430), Beclin1 (No. A11761, ABclonal Technology Co., Ltd., Wuhan, China) and GAPDH (bsm-33033M), CASP9 (bs-20733R), Bcl2 (bs-0032R), ATG5 (bs-4005R, Biosynthesis Biotechnology Co., Ltd., Beijing, China) first antibody at 4°C for 16h. Next, the cells were incubated with the corresponding secondary antibodies (Cowin Biotech Co., Ltd., Jiangsu, China) for 1h at 26°C. After transferring of the protein onto the PVDF membrane, chromogenic agents were added and imaged with a CCD camera-based imager.

Statistical analysis: One-way ANOVA and student's t-test were performed to analyze above-mentioned data by Prism 8.0 software (GraphPad Software Inc., La Jolla, CA). The data were expressed as the standard error of mean (means ± SEM). The photographs were subjected to Imagej (National Institutes of Health). Statistical significance was set as $*p<0.05$ and $**$ $p<0.01$.

RESULTS

Clinical observation of thiram-induced TD: Visible symptoms of weakness and lameness appeared in TD groups (Fig. 1A). Meanwhile, the chickens of TD group showed no desire to stand and were limp, leading to the average weight losses. The situation was eased after the treatment with triptolide in TP group. Feeding behavior of the chickens was improved in TP group and they started to gain weight (Fig. 1B).

Triptolide can improve tibia parameters of TD affected broilers: The results showed that, the weight and the width of the tibia in TD group exhibited slight downward trend and the length of tibia significantly (P<0.01) decreased, while the length of tibial growth plate (P<0.01) significantly increased. The changes of tibial parameters of TD affected broilers were similar to previous studies (Zhang *et al.,* 2020). After triptolide treatment, the length of tibia in the TP group was increased, the length of tibial growth plate declined, almost like the control group. However, the tibia weight was lowest and the width of the tibia was highest in TP group (Fig. 1C, D)

HE staining analysis: The histopathological evaluation revealed that the chondrocytes were regularly and tightly arranged in both proliferative and hypertrophic zones with normal TGPs in the CON group. However, the hypertrophic chondrocytes of broiler in TD group were arranged disorderly and irregularly, and the cells showed no nucleus or nuclear shrinkage. At the same time, the number of chondrocytes in the proliferative area were less than that in the CON group (Fig. 1E, F). After triptolide treatment, improvement in the arrangement and closely packed alignment of chondrocytes were observed in TP group.

Serum biochemical analysis: After thiram administration, the levels of urea (P<0.01), crea and LDH in TD group exhibited an increasing trend compared with the CON group. In addition, AST, T-Bil and D-Bil activity increased while the levels of ALT, ALB and ALP decreased after thiram administration. A rising trend of the contents of Ca and P in TD group could also be seen. Triptolide administration further induced the significant (P<0.01) increase of the levels of urea (P<0.01), crea and LDH compared with the CON group. The AST, LDH and ALP activity were increased along with the levels of ALB (P<0.01) and T-Bil while D-Bil decreased in TP group. The contents of Ca in TD group significantly (P<0.05) decreased in TD chicken (Fig. 1G).

Apoptosis-related genes of chondrocytes analysis by RT-qPCR, western blotting and immunofluorescence: We used RT-qPCR to examine the expression of autophagy-related genes in TGPs. Results of the study suggested that the expression of Bax, p53, CASP8 and CASP9 mRNA decreased. The disposal of TD increased the mRNA contents of APAF-1 and Bcl2. As compared with TD chickens, the treatment of triptolide significantly downregulated the content of Bcl2 (P<0.05). The expression of BAX, CASP8 and CASP9 mRNA also rising but not significantly (P>0.05). Conversely, the expression of p53 and APAF-1 gene mRNA depicted an upregulation in the TP group compared with that in the TD group (Fig. 2A). To assess the protein expression of apoptosis-related genes in tibial, we used western blotting. Our findings indicated that the protein expression of CSAP3, CASP9 and Bcl2 showed a downtrend in TD group. The administration of triptolide reduced the protein level of CASP3 and slightly altered the expression of CASP9 and Bcl2. The CYTC content was similar in each group and there was not significant (P>0.05) difference (Fig. 2B). Immunofluorescence aimed to confirm the effect of TP on CASP3 expressions. The immunofluorescence results (Fig. 2C) showed that, the expression of CASP3 were down-regulated as compared with the CON and TD groups.

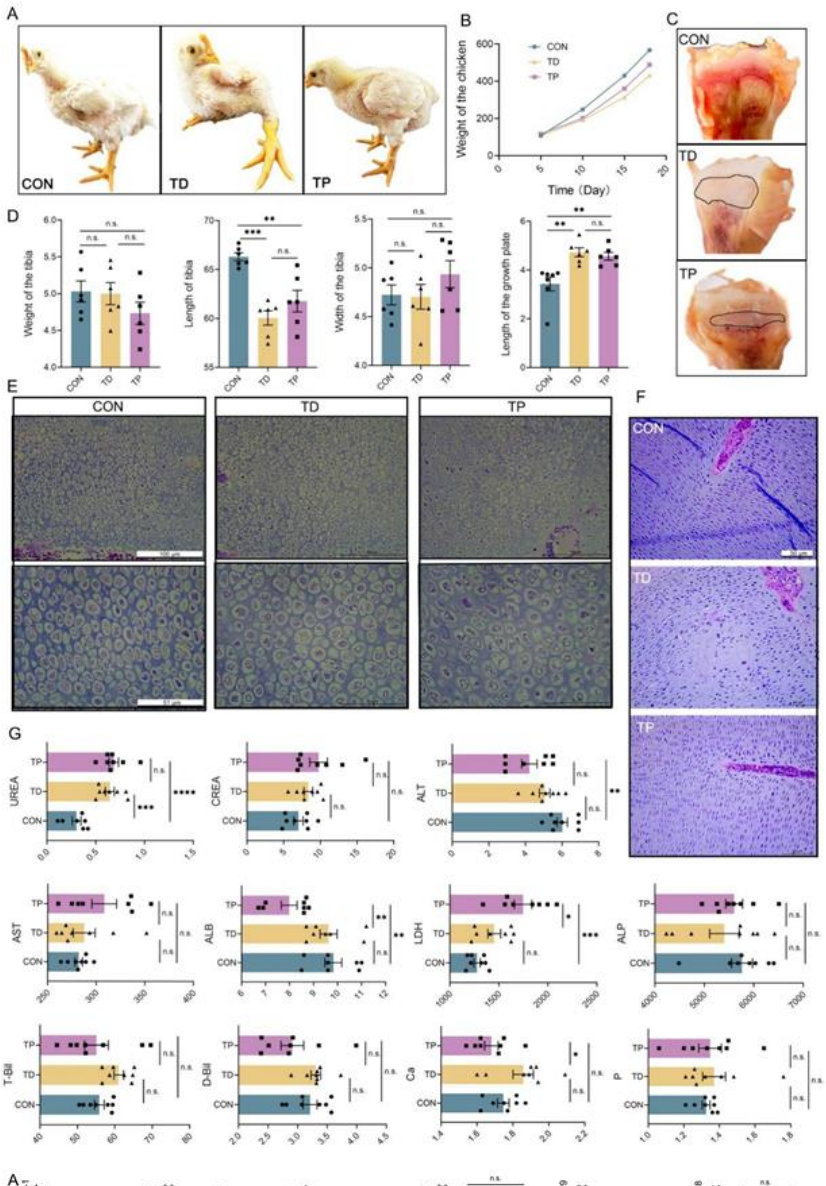

Fig. 1: Triptolide relieves symptoms of TD induced by thiram. A) Clinical symptoms and morphological observation among different groups. B) The body weight of the chicken in the control, TD, and TP groups. C&D) Tibia parameters including the weight, length and width of the tibia, the length of tibial growth plate among different groups. E&F) Histological examination of tibia bones in the control, TD, and TP groups. G) Serum biochemical parameters analysis in the control, TD, and TP groups. All data were expressed in means ± SEM. "*" shows the significancy level among different groups. *p < 0.05, **p < 0.01.

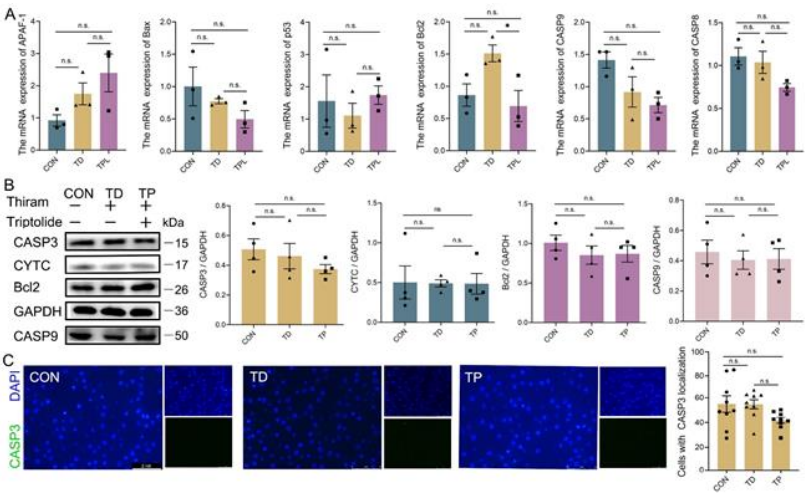

Fig. 2: Triptolide downregulated the mRNA expression of apoptosis-related genes. A) Analysis of apoptosis-related genes (APAF-1, Bax, P53, Bcl2, CASP9 and CASP8) through RT-qPCR. B) The protein levels of CASP3, CYTC, Bcl2, CASP9 in tibial growth plates in three groups via Western blotting. C) Immunofluorescence expression of CASP3 (Magnification 400x). All data were expressed in means ± SEM. "*" shows the significancy level among different groups. *p < 0.05, **p < 0.01.

Autophagy-related genes expression of chondrocytes: The mRNA expression of LC3b, p62, m-TOR and parkin depicted an upregulation and Beclin1 decreased in the thiram-induced TD (Fig. 3A). A trend in the opposite direction was found in the TP group, the mRNA contents of m-TOR and p62 depicted a significant (P<0.05) down regulation approaching to the CON group. The levels of LC3b and parkin showed a downtrend in TP group. The administration of triptolide also lessened the mRNA content of Beclin1 (P<0.05). Analysis of protein expression results of autophagy-related genes by western blotting revealed the expression of ATG5 protein in TP group was the highest, followed by TP group and then CON group, but there was no evidence for a statistically significant difference, and the Beclin1 protein expression have no significant change (Fig. 3B). The immunofluorescence results demonstrated that, the

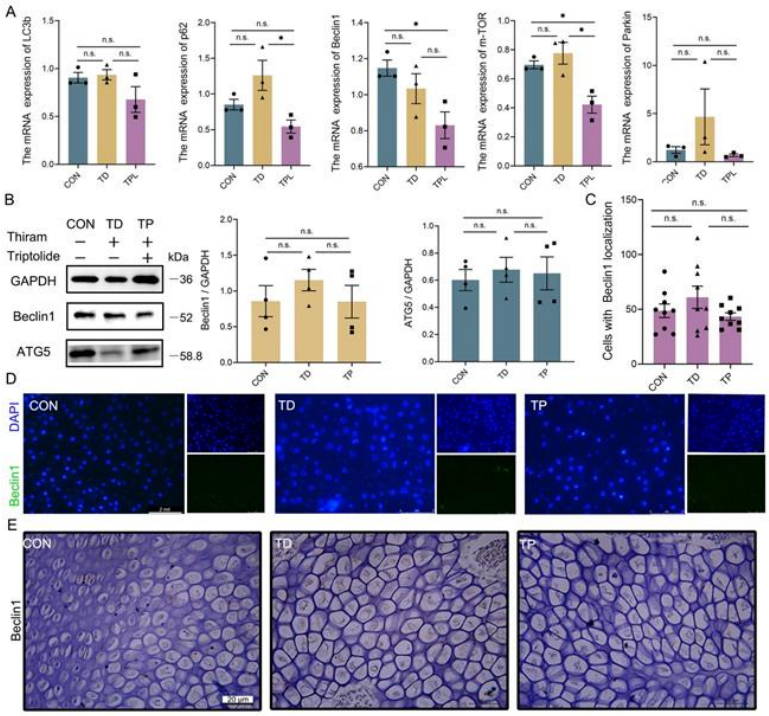

Fig. 3: Effect of triptolide on mitochondrial autophagy pathway. A) Analysis of autophagy-related genes (LC3b, p62, Beclin1, m-TOR, Parkin) through RT-qPCR. B) The protein levels of Beclin1 and ATG5 in tibial growth plates in three groups via Western blotting. C&D) Immunofluorescence expression of Beclin1 (Magnification 400x). E) Immuno-histochemical localization of Beclin1 in control, TD and TP groups. All data were expressed in means ± SEM. "*" shows the significancy level among different groups. *P<0.05, **P<0.01.

highest expression of CASP3 was found in the TD chickens, and the lowest in TP group (Fig. 3C and D). To further confirm this colocalization, the expression of beclin1 antibody in TGPs of the CON, TD, and TP groups was checked by immunohistochemistry (Fig. 3E).

DISCUSSION

The development of TD in poultry is one of the most critical consequences of thiram, which ultimately leading to gait problems in chickens and resulting in substantial losses to the poultry industry (Liu *et al.,* 2021). Triptolide is used in the therapeutic regimens of inflammatory and autoimmune diseases. Researcher found that triptolide can enhance the inhibitory effect of T cells on the differentiation of osteoclast and can enhance the bone to resorb through promoting TGF-β1 and IL-10 to secrete (Xu *et al.,* 2016).

Our study showed that TD reduce the weight gaining ability of broilers. In current research, TD chickens also faced depression, physical discomfort, unwillingness to do an activity, also showed inability to stand and walk. All this seriously affected the performance of broilers. Daily weight gains in the triptolide treatment group increased when compared with TD group, though could not reach the value of the CON group. The overall condition of chickens was improved and they showed willingness to stand after treatment with triptolide.

The development of long bones occurs through the ossification of the chondrocytes of the growth plate. Cellular mechanisms such as continuous cell proliferation, cell enlargement or hypertrophy, and cell clearance appear in the growth plate (Xu *et al.,* 2016). Correlation cases have shown that in TD, abnormal protein excretion and apoptotic behaviors would happen in chondrocytes, which can cause severe cellular damage and eventually lead to a reduced rate of chondrocytes extracellular matrix degradation, thereby limiting the space for bone deposition. In our study, the histological examination of the tibia revealed many cells undergone necrosis and showed nuclear shrinkage and also showed irregular arrangement of the proliferative area or hypertrophic area in the GP of the tibia. After treatment of TD with triptolide, the chondrocytes were arranged in an orderly manner. Previously, Nabi *et al.,* (2016) proved that the treatment of celastrol caused cartilage angiogenesis in TD affected chickens via ruducing Hsp90 expression, restoring the normal GPs of avians (Xu *et al.,* 2016).

High serum levels of urea and creatinine (CREA) provide useful information about the injury of the kidney. Triptolide can alter the morphology and function of the kidney i.e increasing the levels of blood urea nitrogen in serum. Our study is consistent with that the values of creatine in blood serum increased, which indicates triptolide-induced kidney injury (Wang *et al.,* 2009). Their study also showed that an upward trend in LDH release and NAG activity indicates that triptolide induce cytotoxicity in proximal tubule cells (Wang *et al.,* 2009; Bhutta *et al.,* 2022; Kiran *et al.,* 2022). In acute liver damage, AST and ALT releasing after liver parenchymal cell injury are elevated in the serum. Our results showed that triptolide significantly decreased ALT and ALB, and increased AST concentration in chicken serum. The reduction of ALB concentration in serum is usually considered as a marker of the hepatic dysfunction as it is only produced by the liver. Our study found that LDH was released in the serum of chickens in large amounts after treatment with triptolide. ALP activity has a strong association with chondrocyte differentiation, it may also participate in cartilage mineralization. In our study, ALP activity in TD group showed a moderate tendency to decrease as compared with the CON group, and was found slightly up with triptolide treatment.

Many reports demonstrated that apoptosis plays a crucial role in the development and participates in the pathogenesis of diseases (Kashyap *et al.,* 2021). It had demonstrated that apoptosis definitely occurs in maturing

epiphyseal chondrocytes and proposed that a fundamental feature of TD is due to impairment of apoptosis. Research showed that the Bcl-2 expression was increased in TD chickens at day 15, and was upregulated at day 21. The study also found that the VEGF proteins may inhibit the mRNA content of Bcl-2 during the restoration of GP to promote hypertrophic differentiation and apoptosis of chondrocytes in TD lesions (Zhang *et al.*, 2013). Chlorogenic acid reduced the expression of Bax/Caspase-3, and increase Bcl-2 under CD147 signaling axis (Kulyar *et al.*, 2022). Our study found that the concentration of Bcl-2 mRNA showed an upward trend in TD group, and was observably decreased in TP group, but the Bcl-2 expression was lowest in TD group and displayed a slight upward trend in TP group. P53 is known to promote apoptosis. Our findings were consistent with prior findings that triptolide upregulate the concentration of P53 to induce cell apoptosis (Zhao *et al.*, 2020). Previous findings have shown that inhibitory of PI3K-AKT-mTOR pathway increases autophagy and reduces inflammatory responses in articular chondrocytes with OA. The m-TOR expression exhibited a significant downregulation in TD group, but decreased in chickens after treatment with triptolide. Have found that thiram slightly increase the mRNA expression of p62 and parkin (Liu *et al.*, 2022). Reported that TP exerts a therapeutic effect in spinal cord injury through promoting autophagy and found that the expressions of p62 in TP-treated mice declined comparing with DMSO-treated mice (Zhu *et al.*, 2020). Likewise, we found that the mRNA expression of p62 and parkin showed an upward trend in TD group, which exhibited a significant decrease after the treatment with TP. Our findings are also in consistent with that the mRNA content of autophagic gene Beclin1 declined in TD chickens at day 18 (Zhang *et al.*, 2019). However, the inherent mechanism of the downtrend of Beclin1 in our study remained unclear.

Conclusions: The study exhibited that triptolide may protect the chickens against the toxic effects of thiram by regulating mitochondrial autophagy-related genes (m-TOR, P62) and apoptosis-related genes (Bcl2). Triptolide can helps to relive TD to some extent. However, it also poses damage to the liver and kidneys. Furthermore, research on the mechanisms of TD and the principle of triptolide mode of action in TD-treatment still need to be proven.

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Authors contribution: Conceptualization, SZ, ZO, ZT and HZ; data curation, YQ, SC, XG, JX; methodology, SW, KL, JP, LH, YL; project administration, JX, ZT and HZ; writing and review of the manuscript, MK, KM, MUS, ZT and HZ. All the authors have approved the manuscript for publication.

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