RESEARCH ARTICLE

Healing of Growth Plate Cartilage by Hypoxia Inducible Factor-1α Inhibitor Apigenin on Thiram Induced Tibial Dyschondroplasia

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A B S T R A C T

Tibial dyschondroplasia (TD) is a long bone abnormality of many avian species, characterized by distended and unvascularized cartilage of proximal tibiotarsal bone and leads to economic losses and major animal welfare problem in poultry industry. Therefore, this study was designed to investigate the mechanism of hypoxia-inducible factor-1α (HIF-1α) inhibition and antioxidant capacity of apigenin in thiram induced TD. One hundred and fifty broiler chicks were equally divided into 3 groups: control; thiram fed; and apigenin group. The genotypic expression of HIF-1α was analyzed by using quantitative real-time polymerase chain reaction (RT-qPCR) and the protein levels of HIF-1α were determined by western blot analysis. The results showed that HIF-1α mRNA levels were significantly (P<0.05) increased during the course of disease in thiram induced TD birds as compared to control. However, the mRNA levels of HIF-1α was significantly decreased by HIF-1α inhibition factor in apigenin group with diminishes growth plate size and lameness. Moreover, thiram induction also increased the levels of AST, ALT and MDA contents in liver, whereas decreased the antioxidant enzymes and ALP values in thiram group, while these values were found close to normal range in apigenin group as compared to control group in response to apigenin. In conclusion, HIF-1α inhibition can heal lameness and avascularized growth plate in broiler chickens; moreover, apigenin may be efficient through rectifying the liver damage and oxidative imbalance induced by thiram.

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INTRODUCTION

Endochondral ossification is the process in which growth plates propagate longitudinally and link the metaphyseal and epiphyseal region inside the bone. Endochondral ossification comprises proliferation of chondrocytes, production of extracellular matrix (ECM), hypertrophy of chondrocytes, calcification of matrix, vascular invasion, degradation of matrix and finally bone formation (Orth and Cook, 1994).

Tibial Dyschondroplasia (TD) is a kind of long-bone disorder in which growth plate cartilage failed to form bone due to poor vascularization and calcification and cause lameness (Leach and Ornan, 2007). It is one of the most prevailing leg disorders and accounts about 30% of such cases in broiler birds (Pelicia *et al.,* 2012). Avian growth plate normally contains longer columns of chondrocytes with deeper penetration of metaphyseal blood vessels making more vascular growth plate (Pines and Huwirtz, 1991; Pines *et al.,* 2005). TD growth plates are hypoxic as compared to normal growth plate due to lack of vascularization. In TD lesion, transcription factor hypoxia inducible factor 1α (HIF-1α) became up regulated and associated with the severity of disease (Genin *et al.,* 2008; Huang *et al.,* 2018).

Hypoxia is the main factor for the expression of HIF-1α and its accumulation is due to the inhibition of HIF-1a degradation during hypoxia. HIF-1α regulates hypoxic responses and angiogenesis which are necessary for chondrocyte development (Araldi and Schipani, 2010). Role of hypoxia and HIF-1α has been investigated in TD incidence (Herzog *et al.,* 2011; Huang *et al.,* 2017a).

Apigenin (4, 5, 7, -trihydroxyflavone) is a common dietary flavonoid present in vegetables, fruits, spices and herbs belonging to flavone family. It is mostly found in plants such as parsley, celery and dried chamomile flowers. Flavonoids have been investigated due to their antioxidant properties that affect oxygen free radicals which are involved in many diseases (Shukla and Gupta, 2010). Apigenin is non-mutagenic with the property of less toxicity, has particular effects in arresting cell growth and inducing apoptosis in cancer cells (Gupta *et al.,* 2001). Apigenin reduced HIF-1α stability by blocking its binding sites and destabilized HIF-1α protein (Fang *et al.,* 2005).

The main objective of this study was to highlight the effect of apigenin on the expression of HIF-1α and liver functioning in thiram induced TD. The present study was first time using apigenin as an HIF-1α inhibitor property in unvascularized growth plate for the control and treatment of TD in broiler chicken.

MATERIALS AND METHODS

Animal ethics: All animal trials were arranged after the approval and strict guidelines of the Institution Animal Care and Use Committee of Huazhong Agriculture University Wuhan, China.

Experimental design: A total 150 day old broiler chicks were reared under standard conditions. Chicks were allocated into two groups, a control group (n=50) and a thiram group (n=100). Both groups were fed with standard basal diet but thiram group received additionally tetramethylthiuramdisulphide (thiram) @ 50 mg/kg for the induction of disease (Mehmood *et al.,* 2018a, 2018b; Zhang *et al.,* 2018) after three days post-hatch. On day 7, fifty birds were allocated as Apigenin group separated from the thiram group and injected apigenin (Wuhan Dinghui Chemical Co Ltd) intraperitoneally @ 5 mg/kg/day (Liu *et al.,* 2005; Iqbal *et al.,* 2016).

Hematoxylin & eosin (H&E) staining: The tibial bone and liver samples were fixed overnight in 10% formalin in PBS at 4°C and then decalcified in 10% EDTA. These samples were processed in series of graded ethanol, clearing and embedding was done in xylene and paraffin respectively. By using the routine procedure, three sections of 5 μm tissue were stained with hematoxylin & eosin for histological analysis (Bancroft and Gamble, 2008).

Quantification of serum biomarkers and liver antioxidant enzymes: Blood samples were taken by cardiac puncture for evaluation of ALT, AST and ALP. Twenty-five birds from all groups were slaughtered on day 10 and day 14 and after slaughtering liver samples were immediately frozen in liquid nitrogen then stored at -70°C for later analysis of malondialdehyde (MDA) contents, superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) activity. The serum values of ALP,

ALT and AST were measured as unit per liter U/l. Liver MDA contents were calculated in nanomoles per gram wet weight of tissue (nmoles/g) while GSH-Px and SOD and activity were expressed in U per milligram of protein (U/mg protein) by using commercial reagent kits (Jiancheng Biochem Nanjing, China).

Reverse transcription quantitative real time PCR (RT-qPCR): Growth plates of some tibial bones from every group were homogenized in TRIzol reagent and a final volume of 20µl total RNA was transcribed reversely into cDNA using cDNA synthesis kit (TransGen Biotech Co. Ltd, Beijing, China) following suggested protocol by company. The primers for real-time PCR were for GAPDH5′-GCCCAGAACATCATCCCA-3′ 5′-CGGCAG GTCAGGTCAACA-3′ and for HIF-1α 5′-TGAGAGAA ATGCTTACACACAG-3′ 5′-TGATGGGTGAGGAATT GGTTCAC-3′. All PCR reactions were run with the Step One-Plus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in quadruplex by using SYBR® Premix Ex Taq™ kit (Takara, Dalian, China) with following protocol: denaturing at 95°C for 30 sec, 40 cycles at 95°C for 8 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec. Relative expression of gene level was standardized according to the glyceraldehyde 3-Phosphate dehydrogenase (GAPDH) gene expression.

Western blot analysis: The tissue samples (n=6 for each group) were homogenized in ice-cold PBS and incubated at 4°C for 2 h. Samples were centrifuged at 13000 rpm for 10 min to collect the supernatant. Protein concentrations were measured by BCA method (Pierce, Rockford, USA) and were stored at -80°C for further use. The 40μg protein samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gel at 100 mV and were transferred (130 mV for 2h and 20 min) to polyvinylidene fluoride (PVDF) membranes (Millipore, BioSharp, Anhui, China) which were incubated in 5% skimmed milk room temperature for 1h. The membranes were incubated overnight at 4°C with primary anti-HIF-1α mouse monoclonal (1:500 ab 6489; Abcam) antibody. The membranes were washed with PBS (0.01 mol/L, pH 7.4) for 10 min each, then the membranes were incubated with HRP labeled goat anti-mouse secondary antibody (1:4000 dilution) for one hour at room temperature. Bands were visualized by Chemiluminescence (Beyotime Shanghai, China) and image was obtained with luminescent Image Analyzer (ImageQuant, USA).

Statistical analysis: The data were analyzed by one-way ANOVA followed with student *t* test and presented as mean ± standard error of means. The differences were considered statistically significant if P<0.05.

RESULTS

In current study, the mRNA level of HIF-1α gene and liver enzymes were measured before and after apigenin administration in thiram induced TD. Birds of thiram group showing lameness signs after three days of thiram administration and a plug of white avascular mass was found on proximal side of tibia as shown in Fig. 1.
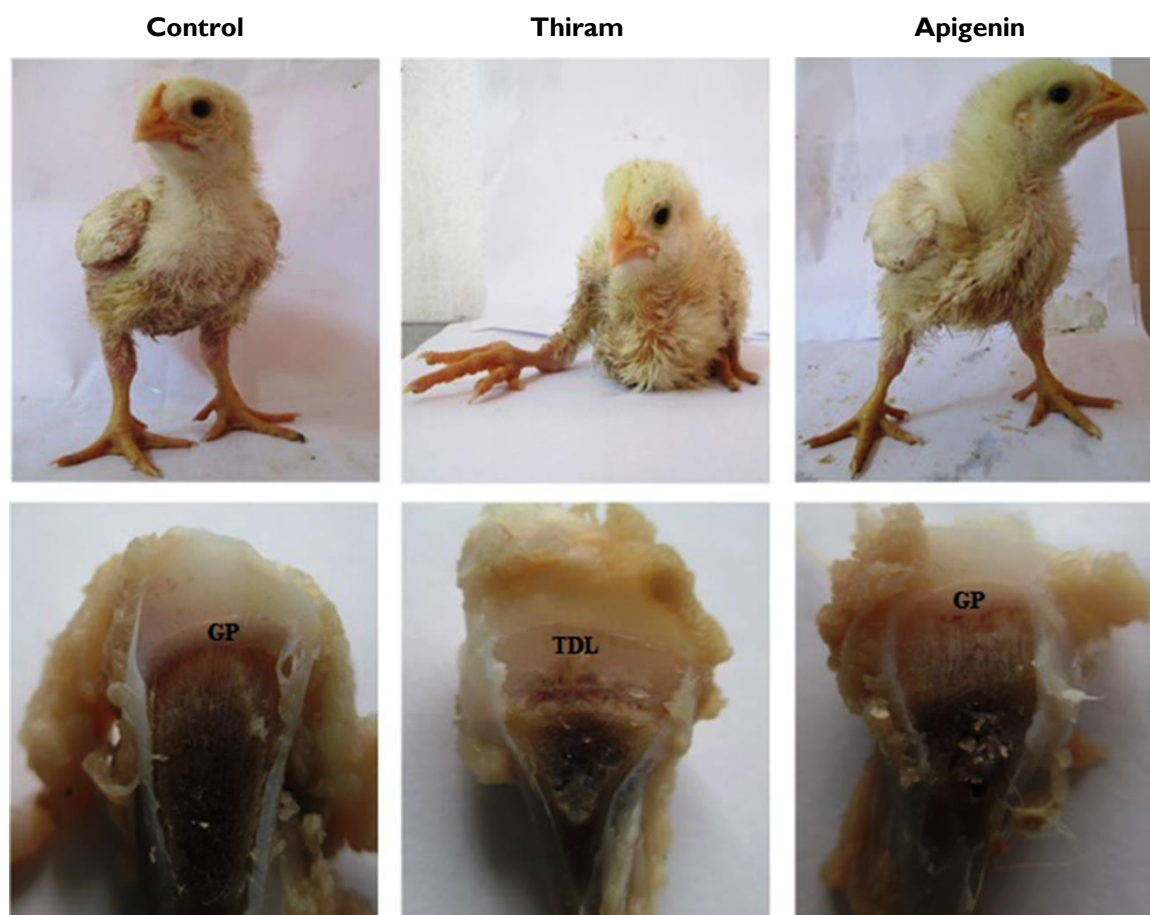
Fig. 1: The Effect of Apigenin on growth-plate width and morphology. Chicks and tibial growth plates were photographed on day 14. Control group with normal growth plate size, thiram-induced tibial dyschondroplasia (TD) group have increased growth plate width while Apigenin group avert growth-plate enlargement after treatment. GP = growth plate; TDL = TD lesion.
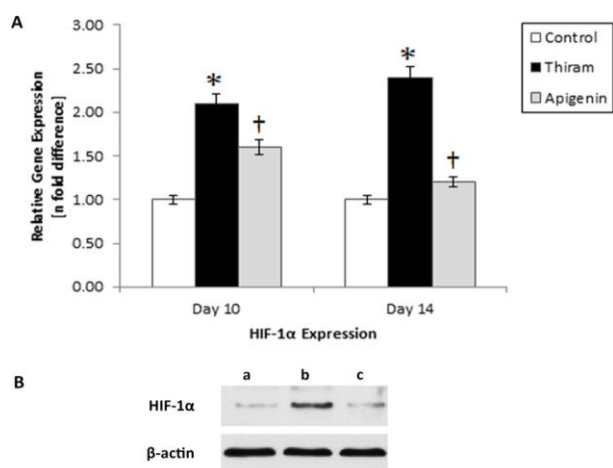

Fig. 2: (A) Effects of inhibition of hypoxia-inducible factor-1α (HIF-1α) in TD affected growth-plate. HIF-1α gene expression levels were evaluated on day 10 and 14 after Apigenin administration. The results are expressed in arbitrary units as the means SE. Control group set to one thus equivalent to the N-fold difference. †* letters show significant difference (P<0.05). (B) Western Blot analysis of HIF-1α in control, thiram-fed and Apigenin treated growth plates at the end of the experiment (day 14) after the Apigenin administration, membranes were also probed with β-actin antibody (n = 6 for each group). Band a, control; band b, Thiram induced; band c, Apigenin treatment.

Histological analysis of growth plate and liver: In control group, chondrocytes were found in the normal columnar arrangement, flattened packed cells, whereas in thiram group chondrocytes were found disorganized,

round in morphology, less vascularized and showed improper distinctions between proliferative and hypertrophic zone. In apigenin group, chondrocytes restored their morphology and columnar arrangement with sprouting blood vessels on day 14 (Fig. 3). There were no injuries in the control group but thiram causesvascular congestion, hepatocytes frequently showed cytoplasmic edema, hemorrhage, hydropic swelling and pyknotic nuclei in liver. After apigenin treatment vascular congestion and hepatic vacuolization became less and hemorrhages decreased in apigenin group (Fig. 4).

Effect of apigenin in TD growth plates: After apigenin intraperitoneal administration, we discovered the relationship between HIF-1α inhibition and TD in chicken growth plates. Expression of HIF-1α gene was up-regulated significantly (P<0.05) in thiram group as compared to control group on day 10 and 14. After apigenin treatment, HIF-1α mRNA levels were down-regulated significantly (P<0.05) in apigenin group as compared to thiram group in chicken growth plates on day 10 and 14 (Fig. 2A). Western blotting analysis further confirmed the expressions of HIF-1α in control, thiram induced and Apigenin groups revealed that increased protein level of HIF-1α in TD afflicted birds as compared to control group. The treatment of TD induced birds with Apigenin led to mark decrease in protein level of HIF-1α and associated equally from chicken growth plate on day 14 (Fig. 2B).
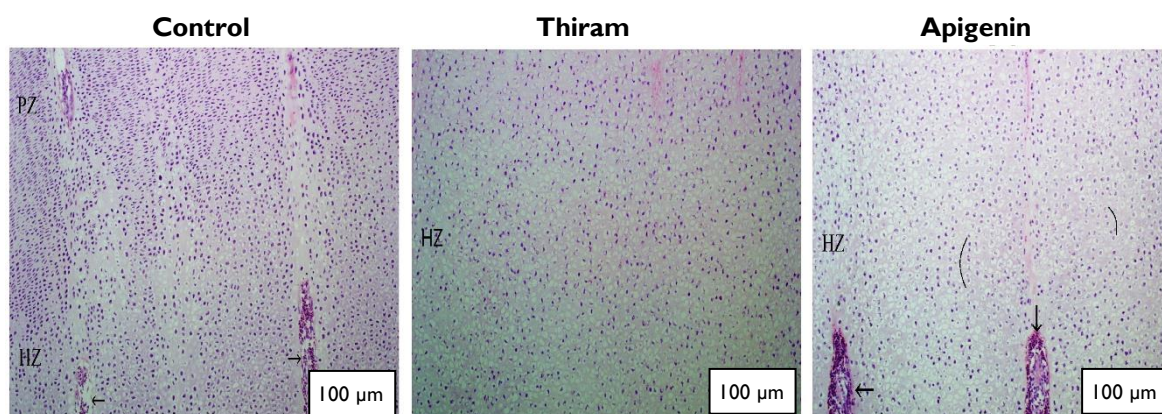
Fig. 3: Hematoxylin and eosin staining of growth plate histological sections of control, thiram and apigenin groups on day 14. Thiram group growth plate was with irregular columnar arrangement of the chondrocytes. Apigenin administration causes the appropriate columnar arrangement with emergent huge blood vessels in the hypertrophic zone. Arrow indicates blood vessels. Columnar arrangements of chondrocytes can be seen in parenthesis. PZ = Proliferative zone, HZ = Hypertrophic zone.
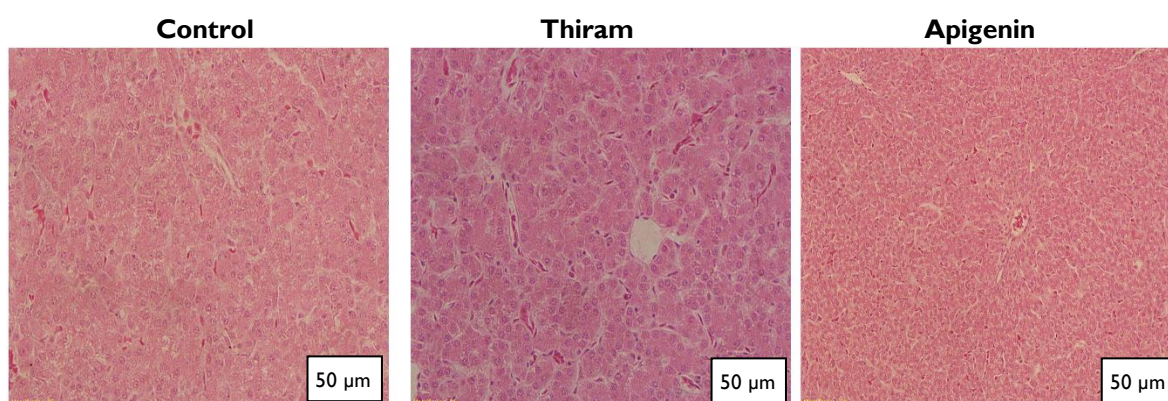
Fig. 4: Hematoxylin and eosin staining of liver histological sections of control, thiram and apigenin groups on day 14. Hepatocyte of control group with normal arrangement of cells while in thiram group, irregular arrangement of hepatocyte, pyknotic nuclei and cytoplasmic edema. After Apigenin treatment less hemorrhages found among hepatocytes, vascular congestion and hepatic vacuolization became less.

Table 1: Effect of thiram (50 mg/kg) in following parameters and recovery through Apigenin (5 mg/kg on day 10 and 14 of experiment (mean±SD)

Parameters/ Days	Control (n=15)	Thiram (n=15)	Apigenin (n=15)
ALT (U/l)			
10	32.56±0.9ᵃ	70.25±1.8ᵃ	59.50±0.9ᵃ
14	42.10±1.2ᵃ	91.85±2.2ᵃ	47.23±0.7ᵃ
AST (U/l)			
10	55.20±1.8ᵇ	125.46±2.5ᵇ	99.45±1.6ᵃ
14	60.35±1.7ᵃ	145.67±2.4ᵇ	68.65±0.8ᵃ
ALP (U/l)			
10	136.45±2.1ᵇ	95.54±1.1ᵃ	125.52±2.4ᵇ
14	152.57±1.9ᵇ	76.34±1.5ᵃ	144.96±2.1ᵇ
SOD (U/mg)			
10	115.31±1.8ᵃ	84.15±0.8ᵃ	105.64±2.2ᵇ
14	130.54±2.5ᵇ	92.7±1.6ᵃ	122.19±1.5ᵃ
GSH-Px (U/mg)			
10	19.85±0.6ᵃ	14.85±0.5ᵃ	17.15±0.5ᵃ
14	23.35±0.7ᵃ	11.87±1.3ᵇ	20.58±0.6ᵇ
MDA (nmoles/g)			
10	25.53±1.7ᵇ	35.64±1.1ᵃ	30.48±0.7ᵃ
14	32.10±1.2ᵇ	43.85±1.5ᵇ	33.45±1.3ᵃ

Superscripts with different letters (ᵃ,ᵇ) in row represent levels of significance (P<0.05).

Effect of apigenin on hepatic serum biomarkers: Current study found increased levels of ALT and AST, while decreased levels of ALP activity in thiram group as compared to control. Conversely, after treatment with apigenin the levels of ALT and AST returned to normal values and marked increase in ALP activity significantly (P<0.05) as shown in Table 1. In our study, we also found significant down-regulation in GSH-Px and SOD activities and up-regulation in MDA contents were found in thiram group as compared to control group showing the abnormal liver functioning, while apigenin treatment restored the level of GSH-Px, SOD and MDA content significantly (P<0.05) as shown in Table 1.

DISCUSSION

TD is welfare and an economical problem in broilers and turkeys growing to their maximum potential and any approach to reduce TD damage is of importance. TD is a disorder of the growth plates at the end of the long bones (Shahzad *et al.,* 2015; Mehmood *et al.,* 2017). Recently it was discovered that up-regulation of HIF-1α is involved in TD probably due to its effect on angiogenesis (Huang *et al.,* 2018; Mehmood *et al.,* 2018b). Avian growth plates contain longer column of chondrocytes as compared to mammalian growth plate and require deeper penetration of blood supply as to reach the proliferative zone (Pines *et al.,* 2005). Normally, avian growth plates are not hypoxic but in case of TD, due to lack of vascularization growth plates became hypoxic. This study describes the effect of HIF-1α inhibitor apigenin on the development of TD lesions in thiram-induced TD in broiler chicks.

The HIF pathway is an essential regulator and important mediator of angiogenesis (Li *et al.,* 2016; Yang *et al.,* 2016). Development of HIF-1α inhibitors through

clinical trials proposed for cancer treatment has been studied previously in humans (Onnis *et al.*, 2009). Among various HIF-1α inhibitors, apigenin was used to inhibit and bind the activity of hypoxia induced HIF-1α expression for combating tumor development (Fang *et al.*, 2005). This study examined the treatment of TD by natural HIF-1α inhibitor apigenin and the protective effect of this medicine as antioxidant in liver oxidative stress of thiram induced TD birds.

HIF-1α is essential for growth and survival of growth plate chondrocytes, while chondrocytes lacking functional HIF-1α have massive cell death in the growth plate which leads to the bone narrow and exhibit less vascularization (Zhang *et al.*, 2012; Yin *et al.*, 2016; Huang *et al.*, 2017b). Previous reports proposed that major regulation of hypoxic responses in chondrogenesis is controlled by HIF-1α (Schipani, 2005). Due to hypoxic environment in TD lesion, an up-regulation of HIF-1α was found in TD, while in normal chick growth plate no hypoxia was observed. Up-regulation of HIF-1α gene expression was dependent on abrasiveness of TD lesion (Genin *et al.*, 2008). An oxygen related gradient was observed inside normal chick growth plate with no hypoxia, while hypoxia is detected in mammalian embryo and has a pivotal role in normal bone growth (Schipani *et al.*, 2001). In current study, mRNA level of HIF-1α gene was significantly up regulated in TD lesion as compared to control on day 10 and 14, while administration of apigenin significantly down regulated the level of HIF-1α in thiram-induced birds. These findings collectively proposed that the cause of TD in broiler chickens is inhibition of tibial angiogenesis, which blocks the supply of nutrients, and chondrocyte death (Yin *et al.*, 2016; Huang *et al.*, 2018).

In our study, thiram was used for inducing TD due to its high regularity and accuracy in production of disease. Thiram is an organic compound which is commonly used as fungicide and pesticide in fields of agriculture (Rath *et al.*, 2007). Thiram had caused hepatic toxicity owing to its main metabolism in liver and up-regulate the level of AST and ALT in broiler chickens (Li *et al.*, 2007; Mehmood *et al.*, 2018a). However, apigenin treatment restored the levels of both ALT and AST by recovering liver damage and lameness. Failure of chondrocyte development and lack of calcification in chicken growth plate are concomitant with reduced serum ALP activity. The alkaline phosphatase (ALP) illustrates hypertrophic chondrocytes and is an indicator of calcification consequently linking with skeletal remodeling (Shahzad *et al.*, 2014).

Thiram amplified the oxidative stress on the liver as an oxidative agent, finally lowering the level of antioxidant enzymes (GSH-Px and SOD) in thiram induced TD birds. Nabi *et al*. (2016a, 2016b) reported increased level of MDA content with decreasing activity of GSH-PX and SOD consequently demolished chicken liver functioning with oxidative imbalance. However, medical therapy recovered liver damage by increasing level of GSH-Px and SOD and decreasing MDA content in liver.

Conclusions: This is the first study to highlight the role of apigenin, which conclude that it is a natural HIF-1α inhibitor for the control and treatment of TD in broiler birds. Furthermore, apigenin exhibited protective effect on liver by restoring oxidative enzymes balance.

Authors contribution: JKL, MKI and FN provided the research idea. MKI, MUR, SH, HZ, LZ, HIA and MI contributed reagents, materials and analysis tools. MKI, KM and YS wrote the manuscript along with handling the revision.

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