



FK228 Ameliorates the Liver Toxicity and Oxidative Stress on Thiram-Induced Tibial Dyschondroplasia in Chicken

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ABSTRACT

Tibial dyschondroplasia (TD) is a disorder of rapidly growing avian species characterized by enlarge avascular growth plate with lesions in proximal tibiotarsal bone. The aim of present study was to investigate the antioxidant capacity of FK228 and role of vascular endothelial growth factor (VEGF) and Flk-1 genes in thiram induced TD. One hundred and fifty broiler chicks were equally divided into 3 groups: control; thiram fed; and FK228 treatment group. Expressions of VEGF and Flk-1 genes were analyzed by real-time quantitative polymerase chain reaction (RT-qPCR) on day 10 and 14 post-hatch. Liver damage caused by thiram was analyzed through levels of antioxidant enzymes (SOD; GSH-Px) and serum biomarkers and the protective effects of the medicine was assessed through these values. Results showed that VEGF mRNA levels were significantly ($P < 0.05$) up-regulated; however, the Flk-1 receptor levels were down-regulated in TD-affected birds significantly ($P < 0.05$) as compared with the control group. Furthermore, thiram induction also increased the levels of AST, ALT and MDA contents in liver, whereas decreased the antioxidant enzymes (SOD; GSH-Px) and ALP values in thiram group, while these values were found close to normal range in FK228 group as compared to control group in response to FK228 treatment. In conclusion, VEGF and Flk-1 genes play an important role in the formation of avascular growth plate, whereas FK228 can heal lameness and avascularized growth plate in broiler chickens. So, it is effectual through rectifying the oxidative imbalance and liver damage.

INTRODUCTION

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 Monsonego-Ornan, 2007). Induction of TD lesion by thiram can be due to interruption in growth plate metabolism and the development of chondrocytes (Rath *et al.*, 2007). Thiram is an organic compound which is commonly used as fungicide and pesticide in fields of agriculture (Rath *et al.*, 2011). Thiram had caused hepatic toxicity owing to its main

metabolism in liver. Thiram exerts a high level of toxicity on endothelial cells inducing death of capillary vessels in the growth plate and interferes with the hypertrophic process resulting in premature death of chondrocytes. Treatment with thiram reduces the concentrations of enzymes and proteins associated with bone development which may be related to the cell death in growth plate (Rath *et al.*, 2005). Normal growth plate development entails cartilage vascularization and mineralization followed by osteogenesis but in TD the differentiation of chondrocytes appear to be abnormal (Rath *et al.*, 2005). Chondrogenesis and osteogenesis are the two major processes that control the rate of bone growth, and vascularization is very critical during exact coupling of these processes in any abnormality or imbalance leads to pathological conditions (Ortega *et al.*, 2010).

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Authors' Contribution

MKI, FN and KM design and perform the experiment. JL and MA contribution of reagents. MAR, MK, SA and AW performed data analysis. MKI, JL and FN Manuscript write up. All authors read and approved the final manuscript.

Key words

Tibial dyschondroplasia, Bone, FK228, Thiram, Liver

Longitudinal bone growth starts where resting chondrocytes of epiphyseal growth plate differentiate into proliferative chondrocytes and form columns of flattened cells and these cells differentiate into hypertrophic zone of epiphyseal growth plate (Genin *et al.*, 2012). Avian growth plate normally contains longer columns of chondrocytes with deeper penetration of metaphyseal blood vessels making more vascular growth plate than the mammalian (Pines and Hurwitz, 1991; Pines *et al.*, 2005). Vascular endothelial growth factor (VEGF) and its receptor affect angiogenesis at various levels and play a major role in the development of TD (Zhang *et al.*, 2013; Huang *et al.*, 2017a).

FK228 is a natural cyclic depsipeptide, approved for the treatment of cutaneous T-cell lymphoma by US food and Drug Administration (FDA). FK228 caused histone acetylation of the VEGF promoter regions, which may contribute to the suppression of VEGF gene expression (Lee *et al.*, 2003; Van der Molen *et al.*, 2011). The present study was designed to evaluate the effects of Fk228 promoting angiogenesis in the avascularized lesions of thiram-induced TD as well as its protective effect on the liver.

MATERIALS AND METHODS

Animal ethics

All animal trials were arranged according to the national legislations designed for animal welfare and 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, one day old broiler chicks were bought from commercial hatchery (Chia Tai Animal Husbandry Co. Ltd, Wuhan, China) and 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 tetramethyl thiuram disulphide (thiram) @ 50 mg/kg for the induction of disease according to Jiang *et al.* (2020) after three days post-hatch. On day 7, fifty birds were allocated as FK228 group separated from the thiram group and injected Fk228 (Shanghai Biochempartner Co., Ltd. Shanghai, China) intraperitoneally @ 0.5 mg/kg/day. FK228 group serve the same diet as thiram group and normal saline was administered to all the control birds. The dose of FK228 was designated based on study using FK228 to treat TD in broiler chicken (Iqbal *et al.*, 2018). Total period of experiment was 14 days.

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 nmoles/g wet weight of tissue while GSH-Px and SOD and activity were expressed in 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 20 µl total RNA was transcribed reversely into cDNA using cDNA synthesis kit (TransGen Biotech Co. Ltd, Beijing, China) following suggested protocol by company. For quantitative real-time PCR, specific primers designated through Primer Express Software v2.0 (Applied Biosystems), based on published *Gallus gallus* sequences. The primers for real-time PCR were for GAPDH 5'-GCCAGAACATCATCCCA-3' 5'-CGGCAGGTCAGGTCACA-3', for VEGF 5'-CGATGAGGGCCTAGAATGTGTGC-3' 5'-AGCT-CATGTGCGCTATGTGC-3' and for Flk-1 5'-GGAGT-TTCCCAGAGACCGAC-3' 5' CAATCCCAAAGGCAT-CAGC-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: initiation with denaturing at 95°C for 30 sec, 40 cycles of amplification 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.

Statistical analysis

The data was carried out among mean values of control and treatment groups by one-way ANOVA is followed by posthoc test and presented as mean ± standard error of means. The differences were considered statistically significant if *P<0.05.

RESULTS

In present study, the gene expressions of *VEGF* and *Flk-1* genes were measured before and after FK228

administration in thiram induced TD on day 10 and 14. 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 Figure 1.

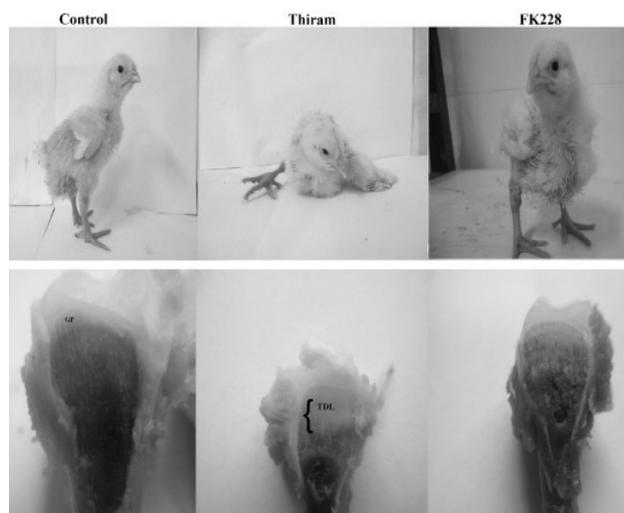


Fig. 1. The effect of FK228 on growth-plate width and morphology. Chicks and tibial growth plates were photographed on day 14. Control group with normal growth plate size, thiram-induced tibial dyschondroplasia (TD) group have increased growth plate width while FK228 group restored growth-plate size after treatment. GP, growth plate; TDL, TD lesion.

Morphological analysis of growth plate

The birds started showing signs of disease after thiram treatment. None of the birds showed diseases in the control group. For the confirmation of TD in the thiram-fed group, proximal tibia bones were dissected lengthwise on days 10 and 14, revealing an opaque avascular cartilage. From day 10, considerable differences were observed between the control and thiram groups. In the control group the tibia bone was ossified with a defined growth plate at the end, while thiram group had developed an opaque avascular cartilage (Fig. 1). In the FK228 group, the lesion started to disappear and the growth plate underwent a recovery process via modelling of the cartilage through resorption and its replacement by bone. On days 10 and 14 most of the area had become calcified and vascularized, leaving behind a small cartilaginous area, showing that the recovery could result in complete healing (Fig. 1). Moreover, improvements were found regarding lameness, body weight and their ability to stand and walk properly (Table II).

Effect of FK228 in TD growth plates

To explain the involvement of *VEGF* and *Flk-1* in normal and TD affected birds, mRNA level of above genes was measured before and after the administration of FK228. During the experiment, increased mRNA levels of *VEGF* and the decreased *Flk-1* receptor levels ($P < 0.05$) were found throughout the course of disease in the thiram fed group as compared with the control (Fig. 2), while FK228 therapy up-regulated the expressions of *Flk-1* in FK228 group (Fig. 3). In present study, increased mRNA levels of *VEGF* were observed non-significantly as compared with the FK228 group on d 10 and 14 ($P > 0.05$).

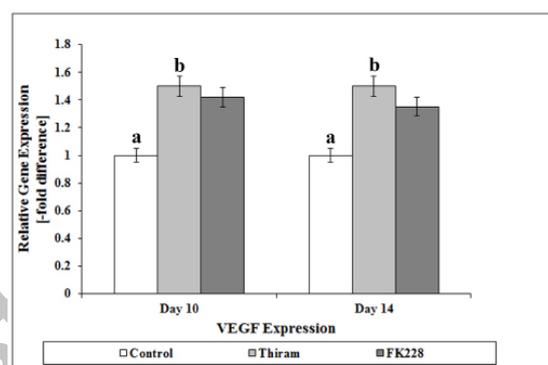


Fig. 2. *VEGF* mRNA expression was analyzed by quantitative real time polymerase chain reaction (qRT-PCR) on day 10 and 14 in avian growth plate of normal, thiram, FK228 treated avian chicks. Data expressed in arbitrary units as the means \pm SE ($n = 6$ for each group). Control group set to one thus equivalent to the N-fold difference. a, b letters designate significant difference ($P < 0.05$).

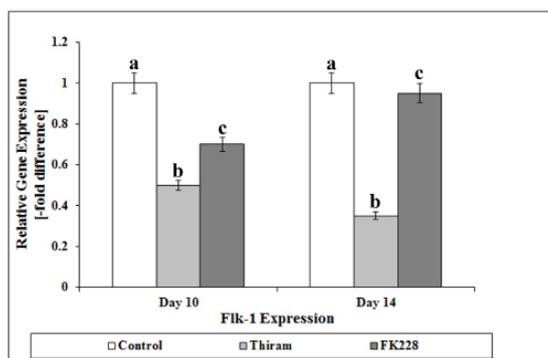


Fig. 3. Gene expression of *Flk-1A* was analyzed by quantitative real time polymerase chain reaction (qRT-PCR) on day 10 and 14 in avian growth plate of normal, thiram, FK228 treated avian chicks. Data expressed in arbitrary units as the means \pm SE ($n = 6$ for each group). Control group set to one thus equivalent to the N-fold difference. a, b, c letters designate significant difference ($P < 0.05$).

Table I. Effect of thiram (50 mg/kg) on following parameters and recovery through FK228 (0.5 mg/kg) on day 10 and 14 of experiment. Values (mean \pm SD) were compared by one way ANOVA.

Parameters	Days	Control (n=15)	Thiram (n=15)	FK228 (n=15)
ALT (U/l)	10	31.58 \pm 0.9 ^a	71.27 \pm 1.8 ^a	55.50 \pm 0.9 ^a
	14	41.20 \pm 1.2 ^a	92.87 \pm 1.2 ^a	46.23 \pm 0.7 ^a
AST (U/l)	10	56.20 \pm 1.8 ^b	124.46 \pm 2.2 ^b	91.45 \pm 1.6 ^a
	14	61.35 \pm 1.7 ^a	147.67 \pm 2.4 ^b	69.65 \pm 0.9 ^a
ALP (U/l)	10	135.45 \pm 2.1 ^b	97.54 \pm 1.3 ^a	122.52 \pm 1.8 ^b
	14	153.57 \pm 1.9 ^b	78.34 \pm 1.2 ^a	146.96 \pm 2.3 ^b
SOD (U/mg)	10	114.31 \pm 1.8 ^a	82.15 \pm 0.8 ^a	102.64 \pm 2.2 ^b
	14	130.54 \pm 2.5 ^b	91.7 \pm 1.6 ^a	121.19 \pm 1.5 ^a
GSH-Px (U/mg)	10	20.85 \pm 0.6 ^a	12.85 \pm 0.5 ^a	17.15 \pm 0.5 ^a
	14	25.35 \pm 0.7 ^a	9.87 \pm 1.3 ^b	22.58 \pm 0.6 ^b
MDA (nmoles/g)	10	27.53 \pm 1.7 ^b	38.64 \pm 1.1 ^a	32.48 \pm 0.7 ^a
	14	33.10 \pm 1.2 ^b	45.85 \pm 1.5 ^b	30.45 \pm 1.3 ^a

*Letters (a, b) represent levels of significance ($P < 0.05$).

Table II. TD incidence, severity scores and body weights of broilers in control, Thiram and FK228 treated groups. The results are expressed as mean \pm SE (n=15).

Parameters	Control	Thiram	FK228
TD incidence (%)	0	70 \pm 5.5 ^a	23 \pm 3.5 ^b
severity score	0	2 \pm 0.2 ^a	1.2 \pm 0.3 ^b
Average body weight (g)	270 \pm 5 ^a	160 \pm 7 ^b	212 \pm 9 ^a

Chicks with scores 0 were designated as normal, 1 as mild pathology, while those with scores 2 were regarded as severe pathology. Values within the same line with different superscripts differ significantly at $P < 0.05$.

Effect of FK228 in liver oxidative stress

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 FK228 the levels of ALT and AST were found close to normal range and marked increase in ALP activity significantly ($P < 0.05$) as shown in Table I. 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 FK228 treatment restored the level of GSH-Px, SOD and MDA content significantly ($P < 0.05$) as shown in Table I.

DISCUSSION

The avian long bone development is a complicated mechanism and is regulated by various clients. These regulating clients interact with each other at certain levels; and any abnormality or imbalance in this mechanism causes pathological changes in bone development. The aberration of growth plate angiogenesis and hypoxic TD lesion may be involved in the pathological mechanisms of TD (Huang *et al.*, 2017b, c). Although, the primary cause of TD is still unknown; however, such pathological changes can be explored to understand better and some medical therapies have been used to treat TD in broiler chicken (Iqbal *et al.*, 2016).

The hypoxic environment in TD growth plates induces the level of VEGF but lesser penetration of blood vessels due to defective chondrocytes in growth plate make it avascular cartilage. Chick growth plates contain longer columns of chondrocytes and require deeper penetration of blood vessels, consequently making more vascular chick growth plates than mammalian (Pines *et al.*, 2007; Herzog *et al.*, 2011). VEGF plays an important role through its receptors; Flk-1 which stimulates blood vessel growth and endothelial cell mitogenesis. VEGF and its receptor Flk-1 are required for bone development and growth, especially in chondrocyte differentiation, membranous ossification and bone angiogenesis (Herzog *et al.*, 2011; Shahzad *et al.*, 2014a; Nabi *et al.*, 2016). According to Velada *et al.* (2011) the increased levels of VEGF in TD growth plates do not stimulate vascularization due to abnormal ECM degradation. Therefore, up-regulated VEGF gene expression cannot exert its function due to arrest in ECM storage sites (Velada *et al.*, 2011). In our experiment, VEGF mRNA expressions were up-regulated in TD-affected birds; however, the Flk-1 receptor levels were down-regulated in these birds. After administering FK228, the mRNA levels of Flk-1 were restored in thiram-induced TD birds and the clinical lameness started subsiding in FK228 group. The up-regulation of VEGF levels and down-regulation of Flk-1 mRNA expressions in TD affected birds were similar to findings from previous studies (Neufeld *et al.*, 1999; Herzog *et al.*, 2011; Zhang *et al.*, 2013; Nabi *et al.*, 2016).

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.*, 2011). 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; Nabi *et al.*, 2016). However, FK228 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 ALP illustrates hypertrophic chondrocytes and is an indicator of calcification consequently linking with skeletal remodeling (Shahzad *et al.*, 2014b).

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.* (2015, 2016) reported increased level of MDA content with decreasing activity of GSH-PX and SOD consequently demolished chicken liver functioning with oxidative imbalance (Nabi *et al.*, 2015, 2016). However, medical therapy recovered liver damage by increasing level of GSH-Px and SOD and decreasing MDA content in liver.

CONCLUSION

The avian long bone development is a complicate mechanism and is regulated by various clients. These regulating clients interact with each other at certain levels; and any abnormality or imbalance in this mechanism causes pathological changes in bone development. Although, the primary cause of TD is still unknown; however, such pathological changes can be explored to understand better and some medical therapies have been used to treat TD in broiler chicken. This study highlights the role of FK228 for the control and treatment of TD in broiler birds. Furthermore, FK228 exhibited protective effect on liver by restoring oxidative enzymes balance.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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