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REVIEW ARTICLE

Role of Oxidative Stress and Antioxidants in Thiram-induced Tibial Dyschondroplasia

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ABSTRACT

Tetramethyl thiuram disulfide (thiram) is an important dithiocarbamate bactericide; it has been widely used for the control of various diseases in fruits, vegetables, seeds and food grains. However, it also causes environmental pollution problems and poses a threat to human health to a certain extent. Thiram induces tibial dyschondroplasia (TD) by causing oxidative stress and antioxidants imbalance in tibial growth plate in poultry. TD is a skeletal abnormality in fast-growing poultry birds. It has been considered an economically important disease in poultry that affects poultry industry by carcass loss at meat processing plant due to decrease in disease resistance, production performance, and carcass quality and induces breast cysts and osteomyelitis worldwide. Oxidative stress is developed due to the imbalance of free radical oxygen, which disrupts the equilibrium state of oxidant and antioxidant tending to oxidation. This review is based on the current research, mainly to explore the relationship and mechanism between tibial dyschondroplasia and oxidative stress induced by thiram, which provides a new theoretical foundation for the further research of the related mechanism.

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INTRODUCTION

Tetramethyl thiuram disulfide (thiram): Tetramethyl thiuram disulfide (thiram) is a broad-spectrum protective low-toxic fungicide, and bactericide, which is wildly used for soil sterilization and seeds treatment (Fig. 1). It has a good control against downy mildew, blight, anthracnose, cereal smut and seedling blight of a variety of crops (Mehmood *et al.*, 2019a). However, the absorption of the thiram into the human and animal's food chain can causes a risk in of position and toxicity. It is irritating to the skin and mucous membrane, though it is a fungicide with low toxicity, and membranes bioaccumulation (Zhang *et al.*, 2019). Thiram has both tumor initiating and tumor-promoting potential and produce a significant decrease in the mitotic index with increasing concentration (Santovito *et al.*, 2012).

Thiram-contaminated feed has been considered a common cause of tibial dyschondrolplasia (TD) in poultry which is related to membrane lipid peroxidation and mitochondrial dysfunction within the cells (Mehmood *et al.*, 2017; Mehmood *et al.*, 2019a). It leads to weight losses and leg deformities in several avian species (Mehmood *et al.*, 2019a). This condition is characterized by oxidative stress injury and apoptosis of chondrocytes, which eventually leads to the accumulation of immature chondrocytes in the chicken growth plate (Waqas *et al.*, 2019; Waqas *et al.*, 2020). Meanwhile, oxidative stress injury of vascular endothelial cells delayed blood vessel penetration, so the chondrocytes in resting and proliferating areas cannot further develop into hypertrophic chondrocyte. The failure of proper endochondral ossification results in the appearance of a visible "white cartilage mass" near proximal end of the growth plate (Zhang *et al.*, 2019).

Normal cartilage development: The process of development of cartilage cells is a highly formalized in tibial growth plate with four development processes, which are divided into resting, proliferative, hypertrophy and calcification zones (Mehmood *et al.*, 2019b; Zhang *et al.*, 2018a). The cells at different periods secrete different proteins. These chondrocytes differ in expression of intracellular enzyme activity, extracellular matrix components, hormone receptors, morphology, growth factors, and secretary capacity (Farquharson and Jefferies, 2000). The proliferation rate of chondrocytes in resting zone is low, and the Sox9, parathyroid hormone-related polypeptides (PTHrP), collagen II (Col II) and Aggrecan are the landmark molecules of cell secretion in this zone (Mizuhashi *et al.*, 2018; Yao *et al.*, 2020). In the proliferating zone, chondrocytes are arranged in columnar and flat shape, with enhanced ability to secrete the cartilage matrix proteins (CMP), and the expression of Aggrecan, collagen (Col XI) and Col II are further increased. Aggrecan and Col X are the matrix components synthesized by chondrocytes in hypertrophy zone (Mehmood *et al.*, 2019b). The surrounding capillaries invade the mineralized tissue area by periosteum in calcification zone, while gergenbauer cells and osteoclasts secrete bone matrix to replace the mineralized cartilage matrix gradually, and finally complete the calcification (Yao *et al.*, 2018). Endochondral osteogenesis mainly occur in hypertrophic chondrocytes via secreting and expressing vascular endothelial cell growth factor (VEGF), attracting the invasion of vascular endothelial cells, and bring prosperous mesenchymal stem cells, osteoblasts and osteoclasts to form bone marrow (Zhang *et al.*, 2018b; Zhang *et al.*, 2013). Meanwhile, matrix metalloproteinases secreted by chondrocytes in hypertrophic stage can accelerate the ossification and extracellular matrix (ECM) degradation of osteoblasts, such as mmp-13, mmp-9, etc. (Zhang *et al.*, 2013).

The morphology of chondrocytes in cartilage tissue vary with the position of distribution. The immature chondrocytes are small in volume and oval in shape, these are located on the surface of cartilage tissue with solo distribution (Zhang *et al.*, 2018a). The long axis is parallel to the surface of cartilage, and the nuclei are lightly stained and oval or round, while the cytoplasm weakly basophilic with varying amounts of lipid droplets. Mature chondrocytes are distributed in the cartilaginous lacunae, which are formed by the division and proliferation of the same metrocyte (Zhang *et al.*, 2018a; Zhang *et al.*, 2018b). Under the electron microscope, chondrocytes have folds and protuberations, and there are developed golgi complex and abundant rough endoplasmic reticulum in the cytoplasm, and few mitochondria. The contraction of chondrocytes was irregular, and there was a large space between the cartilage capsule and the cells under the histological section (Zhang *et al.*, 2018b).

Tibial dyschondroplasia: Tibial dyschondroplasia is a tibiotarsal long bone disease of poultry, which is defined as an avascular and non-mineralized white opaque mass in the proximal growth plate of tibia (Yang *et al.*, 2019) in fast-growing birds. It is an economically important disease because of its devastating effects on poultry industry by carcass loss at processing plant due to low disease resistance, production, and meat quality and affects the carcass grading with breast cysts and osteomyelitis. Besides, it's sub-clinical symptoms which causes difficulties in accurate and timely diagnosis (Groves and Muir, 2017) on other hand it is an animal welfare issues because birds cannot walk and move due to tibia bone deformation, gait disorders, difficulty in standing, reduced growth and lameness, which seriously affects their production performance (Mehmood *et al.*, 2018).

Several phenomena have been reported for the development of poultry tibia dysplasia in chickens (Fig. 2). For example, the fast growth (Duggan *et al.*, 2015; Huang *et al.*, 2018; Zhang et al., 2019), the type of single feed ingredients, the ratio of each nutrient element is unreasonable, the improper feeding and management, and the abnormal local growth factor secretion. The pathogenesis has not been fully understood at present, but there are mainly the following speculations. The fast growing of broiler can have more weight gain burden the leg (Huang *et al.*, 2018), may enhance chest muscles move the center of gravity of the birds towards the front of the body, that changes the biomechanics of long bones (Huang *et al.*, 2017a).

According to the previous studies, when TD occurs, total RBC counts, the level of Hb and Hct (hematocrit) show a reduction by a notable margin and the distribution of blood vessels in the tibial growth plate region is also significantly decreased (Huang *et al.*, 2017a). The RBCs' reduction in the blood is relevant to the apoptosis-related genes' expression in chicken erythrocytes (Wang *et al.*, 2018a). Thus, it can be speculated that abnormal chondrocyte apoptosis is a critical factor for the development of TD. Actually, apoptosis is the result of terminal differentiation of chondrocyte and it is reported the specific relationship between the degree of cartilage damage and chondrocyte apoptosis (Hwang and Kim, 2015).

In addition, the vasculature also deserves our attention; it takes important responsibilities in the development, formation, maintenance and preparation of the endochondral bone in the bone tissue (Ben *et al.*, 2016). Therefore, blood vessel distribution in the tibial growth plate is extraordinary requisite for the bone formation process (Prisby, 2017; Zhang et al., 2018a). Increasing evidence have shown that vascular distribution in the hypertrophic zone of GP reduced and got pale, which further inhibited the bone growth and development (Huang *et al.*, 2017b).

Thiram and oxidative stress: The oxidative and anti-oxidative system is balanced on normal conditions, and what oxidative stress means is that break this equilibrium state tending to oxidation. In fact, cells are equipped with special molecular strategy to control the equilibrium of oxidative stress and maintain a balance of oxidant and antioxidant particles (Cichoz-Lach and Michalak, 2014). Antioxidant enzymes are significant part of the antioxidant system, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX). Besides that, there are non-enzymatic antioxidants substances, like vitamin C, vitamin E and melatonin. Constantly oxidative state causes oxidative damage, which leads to cells dysfunction and cellular behavioral change such as irregular proliferation or accelerated aging, and meanwhile induces apoptotic and autophagy, to eventually causing cell death (Waqas *et al.*, 2019; Huang *et al.*, 2017b) (Fig. 3).

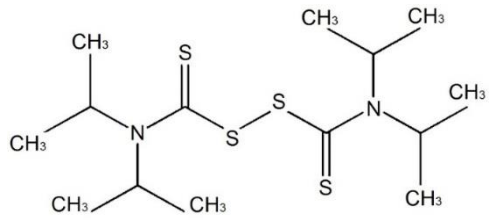

Fig. 1: The structural formula of thiram.

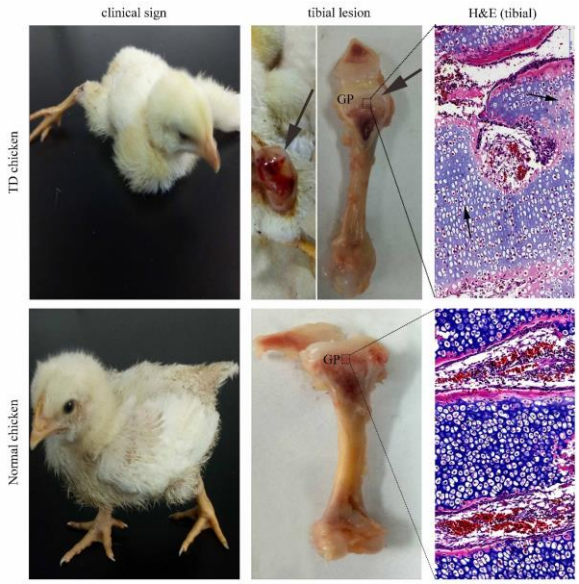

Fig. 2: The different changes of tibial metaphysis in tibial dyschondroplasia chickens. TD chickens show lameness, reduced growth, tibial dyschondroplasia lesion on growth plate (GP), increase in size of GP and H&E staining indicates irregular/less column of cells, cells without nucleus and chondrocytes apoptosis.

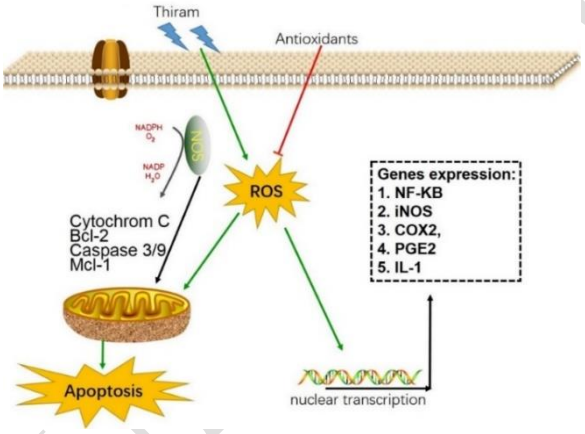

Fig. 3: Model diagram of the influence of thiram on oxidative stress.

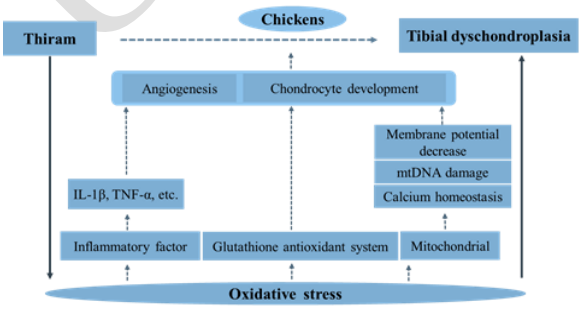

Fig. 4: The influence of thiram on the development of tibia growth plate via oxidative stress in chickens.

Free radical oxygens (ROS) is an active biological molecule that involved in regulating various physiological activities, but it can also cause oxidative damage to cells and tissues if accumulates more than the defense capacity of antioxidants (Lepetsos and Papavassiliou, 2016). ROS produces at a low level by NADPH oxidase in articular chondrocytes, which are helpful for maintaining the cartilage homeostasis. It plays an indispensable role in signaling mechanism of chondrocytes, for example, regulating chondrocytes apoptosis, gene expression, synthesis and decomposition of nuclear extracellular matrix and production of cytokines (Lepetsos and Papavassiliou, 2016).

Thiram has been demonstrated to induce mouse macrophages to produce ROS and increase the level of glutathione (Kurpios-Piec *et al.*, 2015). Thiram causes oxidative modification of cellular components and increase the peroxidation of lipid and protein by increasing ROS and RNS that induces oxidative stress. It weakens the cell's antioxidant capacity, leading to the further oxidatively damage (Salam *et al.*, 2020). According to previous study, thiram induces TD by damaging the liver function and reducing its antioxidant capacity (Li *et al.*, 2016). SOD provides an effective antioxidant protection by specifically eliminating harmful free radicals (O2-) to remove the cell damage which is caused by the free radicals (Younus, 2018). Previous studies indicated that thiram used in chickens could cause the oxidative stress and decreases the contents of SOD and GSH-Px, and increase the levels of MDA (Nabi *et al.*, 2018a). Interestingly, icariin and ligustrazine have been reported to have the function of reducing the incidence of TD by increasing the levels of SOD, T-AOC and GSH-Px with decreasing MDA levels significantly (Zhang *et al.*, 2018a).

TD pathogenesis associated with the abnormal differentiation and accumulation of chondrocytes, and unbalanced chondrocyte morphology also decreased extracellular matrix and level of aggrecan protein in the growth plate (Nabi *et al.*, 2018b). Hsp90 is a chaperone protein that aids other proteins and stabilizes them against stress, which contribute in various processes in angiogenesis, inflammation, tumor and metastasis. It has been demonstrated that Hsp90 is a key factor in the development of TD (Iqbal *et al.*, 2016). Our previous studies have indicated that the Hsp90 widely involved in the regulation of various biological processes in chondrocytes differentiations, bone angiogenesis, and membranous ossification and celastrol restore the normal avian growth plate in thiram-induced TD by inhibiting Hsp90 expression (Li *et al.*, 2020; Nabi *et al.*, 2016). Previous study has indicated that thiram decreases the viability of cell by inhibiting the mitochondrial function (Mehmood *et al.*, 2017). When the cell tissue is damaged by oxidative stress, it destroys the mitochondrial respiratory chain connection so the mitochondrial dysfunction occurs, which induce apoptosis. In addition, the toxic effect of thiram is linked with membrane lipid peroxidation and mitochondrial activity of cell (Mehmood *et al.*, 2017).

In addition, the changes of angiogenesis related factors are considered as an important factor leading to the occurrence of TD in broilers. Thiram induced TD by

changing the expressions of HIF-1α/VEGF/VEGFR signaling pathway (Zhang *et al.*, 2018a). In the early stages of cartilage formation, typical WNT activation inhibit the formation of cartilage by reducing the expression of SOX9 and increasing the expression of anti-chondrogenic gene Twist-1 (Ling *et al.*, 2009). Recent studies have shown that hypoxia may play a key role in the development of TD in broilers, which plays a significant role in angiogenesis (Huang *et al.*, 2017a), chondrogenesis modulation, and cell apoptosis (Yin *et al.*, 2016).

Oxygen free radical imbalance and chondrocyte development: Oxidative stress is the main damaging effect of thiram-induced cartilage dysplasia (Fig. 4). Articular cartilage tissue cells maintain the homeostasis of oxygen free radicals in the microenvironment by regulating HIFs to ensure the specificity of their own structure and function (Tsuchida *et al.*, 2014). Oxygen free radicals generated by mitochondrial electron transfer are beneficial to maintain the redox balance of cell glycolysis to ensure the dynamic balance of chondrocyte metabolism (Martin and Buckwalter, 2012). Active oxygen causes chondrocyte damage by increasing the production of inflammatory mediators such as IL-1β and TNF-α, which can directly assault proteoglycan, matrix protein and membrane protein (Hosseinzadeh *et al.*, 2016). Previous studies have shown that high level of ROS induce the degeneration of cartilage and cause necrosis by lysing collagen, aggregating proteoglycans and activating matrix metalloproteinases (MMPs) (Henrotin and Kurz, 2007).

Inflammatory factors such as TNF produced by oxidative stress can induce more peroxidation of chondrocytes (McCord and Edeas, 2005). Reactive oxygen plays an important role in initiating apoptosis. Previous studies have shown that too high ROS will increase the damage of mtDNA in chondrocytes and thus increase the sensitivity of chondrocytes to TNF and IL-1β (Kim *et al.*, 2009). It can be seen that imbalanced oxygen free radical greatly reduces the ability of chondrocytes' self-repairing. In addition, mic-RNA level will change significantly once the balance of oxygen free radicals was break. For example, overexpression of PTHrP delays the maturation of chondrocytes and the formation of blood vessels (Duan *et al.*, 2019). Mic-RNA can suppress the inflammation by inhibiting the activity of NF-KB and affecting the expression of its target genes (Yang *et al.*, 2014). However, for patients with osteoarthritis, the imbalance of oxygen free radicals weakens this inhibitory effect; on the contrary, it will amplify the NF-KB signal, resulting in persistent inflammation.

Oxidative stress causes mitochondrial dysfunction, impaired mitochondrial DNA integrity and repair ability, and induces the occurrence of autophagic apoptosis and cartilage degradation (Kim *et al.*, 2000) . Normally, chondrocyte proliferation and apoptosis are in a dynamic balance circumstances; however, excessive apoptosis of chondrocytes appeared in TD chickens, thus the formation of cartilage is destroyed (Zhang *et al.*, 2020). At the same time, oxidative stress makes the chondrocytes more susceptible to oxidant-mediated cell death (Carlo and Loeser, 2003). In contrary imbalanced oxygen also disrupts the mitochondria Ca^{2+} homeostasis, the mitochondrial respiratory connection is destroyed by the increasing accumulation of mitoCa^{2+}, mitochondrial dysfunction occurs, and apoptosis is induced. ROS promotes the release of cytochrome C, which is a key link in apoptosis. In the presence of dATP, cyt-C binds to Apaf-1 to activate caspase-9. Caspase-9 is a key link of caspase family to regulate apoptosis; it further activates other caspase protein such as caspase-3 (Rasheed and Haqqi, 2012).

Oxygen free radical imbalance and vascular endothelial cell apoptosis: Vascular endothelial cell apoptosis is one of the important factors of cartilage dysplasia (Mehmood *et al.*, 2017). Wang et al. Found that miR-124 can induce apoptosis of cerebral vascular endothelial cells by down-regulating the PI3K/ AKT signaling pathway and promoting the generation of ROS (Qamar *et al.*, 2019; Wang *et al.*, 2018b). oxLDL causes DNA methylation, including nuclear DNA and mitochondrial DNA (Zeng *et al.*, 2019). DNA hypermethylation leads to mitochondrial dysfunction, and it has been reported that oxLDL induces pyrolysis of vascular endothelial cells through miR-125p / TET2 channel (Chipuk and Green, 2006). Mitochondria are directly associated to cell death, and mitochondrial membrane damage potential is considered to be the earliest cascade of apoptosis. Free radicals activate nuclear transcription factors (NF-KB), NRF2, P53, and these transcription factors are all involved in the activation of apoptosis. P53 can interact with anti-apoptotic Bcl-2 family, promoting apoptosis proteins to induce changes in mitochondrial outer membrane permeability. Due to this change in mitochondrial outer membrane permeability, resulting in the oligomerization of the apoptotic protease activating factor APAF1 to form an apoptotic body, which aggregates and activates procaspase-9 and then caspase -3, caspase-7, that is sign of apoptosis (Desoti *et al.*, 2012).

As thiram induced TD is characterized by avascular cartilage, consequently neovascularization is an important physiological mechanism for the recovery of TD. The mobilization of vascular endothelial cells from bone marrow is beneficial to the formation of blood vessels after tissue ischemia (Carmeliet, 2000). This process depends on growth factors, and normal physiological levels of ROS due to tissue ischemia, which involves in cell proliferation and migration, thereby promoting the formation of new blood vessels (Pearlstein *et al.*, 2002). Vascular endothelial growth factor (VEGF) is considered as the most specific factor in inducing angiogenesis by promoting division and chemotaxis, and stimulates the occurrence and growth of blood vessels. Previous studies have definite that the oxidative stress can promote the expression of VEGF (Frezzetti *et al.*, 2017). Cheng et al., found that hypoxic conditions accelerate the release of ROS and the expression of VEGF and HIF-1α; meanwhile, VEGF increased the generation of ROS and enhanced the stimulation of ROS on angiogenesis (Cheng *et al.*, 2019).

Prospection: The mechanism of tibial dyschondroplasia is very complex, involving many genes and proteins.

Achondroplasia (ACH) is an autosomal dominant genetic disease and one of the common congenital dwarfisms, which increased the ratio of birth defect in newborns, while genetic diagnosis is the most accurate method to diagnose ACH. There is no effective treatment for ACH currently, so prenatal diagnosis is particularly important. As the present research shows, exon 10 cDNA 1138 G> A point mutation is the most common type of ACH. In this approach, we can acquire some implications that we can target the gene-level research, screening the genes involved in the growth and development of chondrocytes which firmly express in the clinical phenotype in the study of dysplasia of avian cartilage and figure out how these genes affect the transmission of signals between cells, such as cell apoptosis.

Based on the current research progress, it is not difficult to conclude that oxidative stress plays role in the formation of thiram-induced tibial dyschondroplasia. Mitochondria are important pivots and the energy factories which participate in mediating many metabolic activities. However, oxygen free radicals have been reported to cause direct damage to mitochondria, changing the permeability of the outer membrane of mitochondria leading to mitochondrial dysfunction, and inducing mitochondrial DNA mutations to accelerate cell aging which affect the normal growth and development of chondrocytes. Therefore, individuals with excellent genes can be selected and propagated in the production practice, which greatly reduces economic losses. Furthermore, we can attempt to explore the pathogenic mechanism of thiram-induced tibial dyschondroplasia from the perspective of mitochondrial damage consequences and use targeted therapy for the treatment and control of tibial dyschondroplasia.

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Authors contribution: HZ and ZXT conceived and supervised the review; BXL and YL analyzed data and wrote the manuscript; KM and FN, SA, TR, MF, MA revised the manuscript.

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