Thiram-induced tibial dyschondroplasia: a model to study its pathogenesis and prevention

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Tibial dyschondroplasia (TD) is a metabolic cartilage disease that causes major leg problems in poultry. We have developed a model to induce TD at high incidence and severity by feeding week-old broiler chickens diets containing 50-100 ppm thiram for two days that is amenable to study the mechanisms of the disease and screen for factors that may prevent it. The objectives for this study were to understand what causes avascularity of TD lesions and to determine if certain micronutrients added to their basal diets may protect the chickens from this disease. Week-old broiler chickens were fed 100 ppm thiram and monitored for the expression of selective genes associated with vascularization and cell survival at 48 and 166 h after feeding thiram. RT-PCR and capillary electrophoresis was used to determine the expression of vascular endothelial growth factor (VEGF), its receptors (VEGFR), and anti-apoptotic protein BCL-2 genes. The results showed maximal suppression of VEGFR and BCL-2 genes at 48 h following thiram feeding, when only sporadic capillary endothelial cell death was observed by histochemical methods. Except for BCL-2 these suppressions were not evident at 166 h when the lesions are visually discernible with chondrocyte death apparent histologically and histochemically. Further analysis suggested that the dead cells in the lesions were those affected early during thiram treatment. The avascularity of the TD lesion seems to be related to the early death of capillary endothelial cells which express VEGFR through which VEGF, produced by hypertrophic chondrocytes, transduce signals for angiogenesis. The thiram-initiated cell death was also evident by the suppression of BCL-2 gene. In order to test the potential of this model as a bioassay for nutrients that may protect birds against TD, selective micronutrients were added to the diets and the birds were subjected to TD-induction with 50 ppm thiram and examined for disease incidence and severity on day 15. Of the tested dietary supplements that included vitamin A, folic acid, trace minerals, and different forms of vitamin D, only folic acid at 5 times its NRC recommended concentration protected chickens against TD. These studies provide an example of how thiram-induced TD can be exploited to study the mechanisms of this economically important poultry metabolic disease.

Keywords: tibial dyschondroplasia; thiram; angiogenesis; folic acid

Introduction

Tibial dyschondroplasia (TD) is a major leg problem in meat-type poultry which results from incomplete bone formation at the proximal end of tibia and tibio-tarsal bones (Leach and Nesheim, 1965; Whitehead 1995; Farquharson, 2002; Rath *et al*., 2004; Pines *et al.,* 2005). Controlling TD requires a better understanding of its etiology and the mechanism of the disease in order to rationally prevent the disease through nutritional or management approaches. With sporadic incidence and indeterminate timelines of naturally occurring TD, it is difficult to understand the mechanisms of its pathogenesis. Experimental models that simulate the production of the disease in a rapid and repeatable manner provide an opportunity to follow TD's initiation and progression and are also useful as bioassay models to screen for the factors that may prevent the disease. Dithiocarbamate

pesticides are environmental chemicals widely used in agriculture and many household products as fungicides, herbicides, insect and pest repellants (US EPA, 2001). Chronic exposures to several dithiocarbamate pesticides such as tetramethyl thiuram disulfide (thiram) or disulfiram have been reported to boost the incidence of TD in chickens (Vargas *et al*., 1983; Edwards, 1990). By feeding week-old broiler chickens diets containing 50-100 ppm thiram for a day or two, we have been able to induce TD at a very high incidence and severity rate making it amenable to study the mechanism of the disease and using it as a bioassay screen (Rath *et al*., 2005; 2006). Since some hallmark features of TD lesions are the avascularity of the cartilage plug with extensive death of chondrocytes (Rath *et al*., 1998; 2005), one of the objectives of our study was to determine if thiram alters the expression of genes associated with angiogenesis and cell survival. Using selective candidate genes associated with angiogenesis, vascular endothelial growth factor (VEGF), its receptors (VEGFR), and an anti-apoptotic protein BCL-2 (Eguchi *et a*l., 1992), we examined their expression using an early and a later time point following treatment with thiram.

For the second objective we examined the suitability of this experimental model to evaluate the efficacy of selective micronutrients with osteotropic and cytotropic efficacies that may provide protection against TD. We used vitamin D, vitamin A, pyridoxine, folic acid, and trace minerals to examine their potential to protect against TD induced by thiram. Vitamin D and some of its metabolites have been shown to be beneficial against TD induced by feeding chickens diets containing low Ca and high P diets (Edwards, 1990; Rennie and Whitehead, 1996; Mitchell *et al.,* 1997; Ledwaba and Roberson, 2003; Whitehead *et al.,* 2004).

Materials and methods

All experiments were conducted under Institutional Animal Care and Use guidelines. Day-old broiler chicks were raised in Petersime batteries with free access to chick starter diet (NRC, 1994) and *ad libitum* water under a constant light period of 23 hours. For gene expression experiments, a group of birds were subjected to induction of TD by including tetramethylthiuram disulfide (thiram) in their diets for a period of 48 hours on days 8 and 9 post hatch as previously described (Rath *et al.,* 2004). The control birds received normal diets only. The growth plate cartilage tissues from 4-5 individual chicks in each control or thiram-fed groups were harvested at 48 and 166 h of feeding diets with thiram. RNA, extracted using TriReagent (Sigma Chemical Company, St. Louis, MO), was subjected to DNAse digestion with a column procedure (Qiagen, Valencia, CA) and quantified using Ribogreen reagent (Molecular Probe, OR) (Rath *et al.,* 2005). Identical quantities of RNA from all birds were subjected to reverse transcription (RT) using an Ambion Retroscript kit (Ambion, Austin, TX) to synthesize cDNA. Equal aliquots of cDNA was polymerase chain reaction (PCR) amplified using a multiplex kit (Qiagen, Valencia, CA) containing five sets of primers corresponding to vascular endothelial growth factor (VEGF), two VEGF receptors (VEGFR1/Flt-1 and VEGFR2/Flk-1), anti-apoptotic protein BCL-2, and 18S RNA (Table 1). The universal primers for 18S gene were obtained from Ambion (Austin, TX) as a part of a kit containing a competimer that is used to control amplification of 18S RNA. The primer to competimer ratio was maintained at 3:7 based on preliminary studies as per the suggestion of the manufacturer. The PCR products were analyzed and quantified using a P/ACE capillary electrophoresis system equipped with a Laser-Induced Fluorescence detector (Beckman-Coulter Company, CA) as previously described (Rath et al., 2005). The changes in the expression of genes were calculated relative to 18 S amplicon.

In the second objective we explored the potential of this model as a bioassay to evaluate the efficacy of micronutrients to counter the induction of TD. The chicks for these studies were maintained with starter diets (NRC, 1994) fortified with supplemental levels of vitamin D or its active metabolite 1, 25 $(OH)_2$ D3 that has been shown to be beneficial in models using low Ca and high P diets to induce TD (Edwards, 1990; Rennie and Whitehead, 1996; Mitchell *et al.,*1997; Ledwaba and Roberson, 2003; Whitehead *et al*., 2004).Vitamin A, pyridoxine, folic acid, and some selective trace minerals were also tested using concentrations above and over the amount present in the normal diet since several of these are known to have beneficial effects in maintaining skeletal physiology (Nilsson

et al., 2005). On day 7 all chickens were feed withdrawn overnight as described earlier (Rath *et al*., 2004) and the birds in each feed group were divided into two sub groups one receiving the diet-specific control feed the other receiving same feed with 50 ppm thiram for 48 hours. After 48 h all birds in each feed group received their stipulated diets until necropsy on day 16 when the TD index was determined examining the proximal tibia. Quantitative data were evaluated using GLM procedure and the means separated using Duncan's Multiple Range test (SAS Institute, 1994). A P value ≤ 0.05 was considered significant.

Results and discussion

The results of the gene expression studies are shown in Table 1. Thiram did not negatively affect VEGF expression at any time but significantly reduced the expression of both of its receptors and the anti-apoptotic protein BCL-2 at 48 h. Except for BCL-2 the effects of thiram were not statistically different at 166 h following treatment (Table 1). Angiogenesis, a process of forming new vessels from pre-existing blood vessel (Folkman and D'Amore, 1996), is essential to endochondral bone formation (Gerber et al., 1999). It is particularly relevant to avian growth plate because unlike in mammals where it is completely avascular, the avian growth plate is vascularized with sporadically interspersed capillary vessels in all regions of the growth plate (Pines and Hurwitz, 1991). Many growth factors and enzymes are involved in the process of angiogenesis and vascularization (Carmeliet and Jain 2000), foremost among them is vascular endothelial growth factor (VEGF) that plays a major role in the growth plate development (Gerber et al., 1999; Ferrara 2004). Angiogenesis also leads to cartilage remodeling and osteogenesis (Maes et al., 2000; Provot and Schipani, 2005). The hypertrophic chondrocytes are principal sources of VEGF in growth plate (Carlevaro et al., 2000). VEGF signaling for angiogenesis however, is mediated via specific receptors named Flt-1 or VEGF receptor 1 (VEGFR1) and Flk-1 or VEGF receptor2 (VEGFR2) that are located in the endothelial cells of blood vessels (Gerber *et al.,* 1999; Ferrara, 2004). The results of our experiments show that the expression of VEGF is not decreased by thiram although such treatment invariably produced TD in ≥90% of birds (Rath *et al*., 2004). On the contrary, for unexplained reasons, there was an increase in the expression of VEGF gene seen at 48 h in birds that received thiram, and is consistent with our earlier observation (Rath *et al.,* 2005). Though thiram significantly reduced the expression of both VEGFR1 and VEGFR2 genes at 48 h its effects were not statistically different from control diet fed birds at 166 h. Our observation using histological and histochemical studies suggest that death of capillary vessels is possibly an early event that leads to the arrest of angiogenesis and subsequent cartilage remodeling, and osteogenesis (Rath *et al*., 1998; 2004, 2005). Despite normal to high expression of VEGF, the dwindling expressions of its receptors and the anti-apoptotic protein BCL-2 possibly lead to an arrest of angiogenesis. The failure of capillary vessels due to endothelial cell death may lead to a progressive increase in chondrocyte death and the arrest of tissue remodeling, and finally to the retention of an avascular cartilage plug containing with dead chondrocytes. However, we can not explain why thiram did not decrease VEGF expression although it may be possible to explain why no down regulation of VEGF and VEGFR genes at later time 166 h was evident when there was massive chondrocyte death. The apparent paradox could relate to procedural bias. RNA is contributed both qualitatively and quantitatively by the living population of cells; therefore, is largely obtained from those cells that are not affected by thiram at 166 hours post treatment. Consequently, the changes in gene expression are not seen at this time despite the presence of massive death in earlier exposed cells. Both thiram and disulfiram have been shown to be anti-angiogenic in other experimental models (Marilkovsky, 2002). By causing death of endothelial cells and capillary vessels thiram appears to block angiogenesis thereby precluding growth plate remodeling and osteogenesis.

The second set of experiment was designed to test the potential of this model to bioassay the efficacy of selective micronutrients against TD induction by thiram. Neither vitamin D, nor its metabolite 1, 25 $(OH)_2$ D3, or Vitamin A, E, pyridoxine, and several trace metals (zinc, copper, iron) added in excess of the normal NRC recommended concentration were able to prevent TD (*data not shown*). However, folic acid at concentration of 1.6 mg/kg (~5 times the NRC required concentration) was able to reduce the incidence of TD (*Figure 1*) suggesting that it may be possible to judiciously design and screen for nutrients that may reduce the development of tibial dyschondroplasia in poultry.

In conclusion, the thiram-induced TD model can be exploited to understand the mechanisms of its pathogenesis and screen for nutrients that may control this economically important poultry metabolic disease.

Table 1: Changes in the expression of selective candidate genes*associated with growth plate vascularization and cell survival at 48 and 166 hours after feeding 100 ppm thiram.

Genes Primers	48 h (n=5)		166 h (n=5)	
	Control	Thiram	Control	Thiram
Vascular Endothelial Growth Factor **F:** ggaagcccaacgaagttatc **R:** aacccgcacatctcatcag	1.56 ± 0.06^{c}	1.91 ± 0.11^{a}	$1.61\pm 0.14^{b,c}$	$1.65\pm 0.07^{a,b,c}$
Vascular Endothelial Growth Factor Receptor 1 (Flt-1) **F:** gcaggcagcttgaaagaaac **R:** gctggcctttcatgactctc	0.88 ± 0.06^{a}	0.56 ± 0.03^{b}	$0.73\pm 0.09^{a,b}$	0.67 ± 0.12^{b}
Vascular Endothelial Growth Factor Receptor 2 (Flk-1) **F:** caccatggtctgttccagtg **R:** ccatggctgcagtctctgta	0.80 ± 0.04^{a}	0.49 ± 0.04^{b}	$0.65\pm 0.04^{a,b}$	0.49 ± 0.13^{b}
BCL-2 **F:** gcaggcagcttgaaagaaac **R:** gctggcctttcatgactctc	0.61 ± 0.03^{a}	0.38 ± 0.03^{b}	0.60 ± 0.04^{a}	0.35 ± 0.08^{b}

* The results are ratios of specific gene amplicon relative to 18S amplicon. Values in a row with no common superscripts denote significant differences ($P\leq 0.05$). F= Forward primer; R= Reverse primer

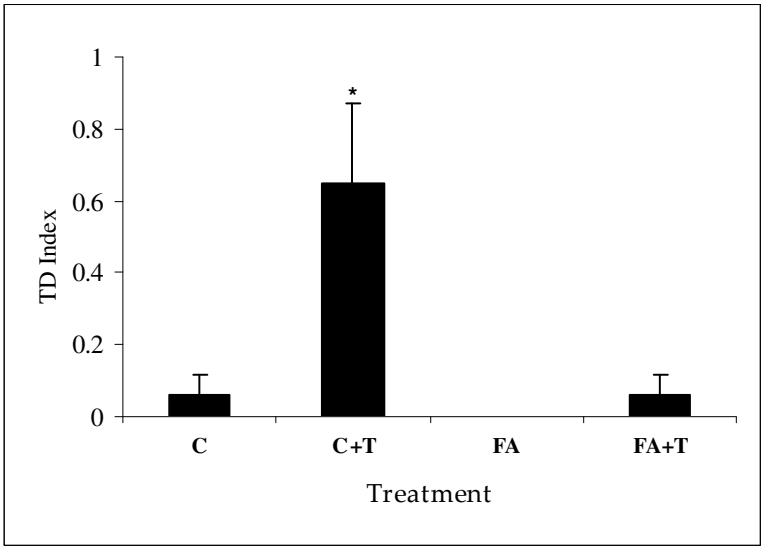

Figure 1: TD indices of control and extra folic acid-fed birds with or without thiram treatment (n=18). C= Control basal diet; C+T = Basal diet with 50 ppm thiram fed for two days; FA= Basal diet with extra folic acid; FA+T = Basal diet with extra folic acid and 50 ppm thiram fed for two days. * denotes significant differences from other treatment groups ($P\leq 0.05$).

References

CARLEVARO, M.F., CERMELLI, S., CANCEDDA, R., and CANCEDDA, F.D. (2000) Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. *Journal of Cell Science* **113:** 59-69.

CARMELIET, P. and JAIN, R.K. (2000) Angiogenesis in cancer and other diseases. *Nature* **407:** 249-257.

EDWARDS, H.M, JR. (1990) Efficacy of several vitamin D compounds in the prevention of tibial dyschondroplasia in broiler chickens. *Journal of Nutrition* **120:** 1054-1061.

EDWARDS, H.M. JR. (2000) Nutrition and skeletal problems in poultry. *Poultry Science* **79:** 1018-1023.

EGUCHI, Y., EWERT, D.L., and TSUJIMOTO, Y. (1992) Isolation and characterization of the chicken bcl-2 gene: expression in a variety of tissues including lymphoid and neuronal organs in adult and embryo. *Nucleic Acids Research* **20:** 4187-4192.

FARQUHARSON, C. (2002) Tibial dyschondroplasia: a growth plate abnormality caused by delayed terminal differentiation, in: *The Growth Plate* (I. M. Shapiro, B. Boyan, and H. C. Clark-Anderson, eds), pp 201-211. IOS Press, Amsterdam.

FERRARA, N. (2004) Vascular endothelial growth factor: basic science and clinical progress. *Endocrine Review* **25:** 581-611.

FOLKMAN, J. and D'AMORE, P.A. (1996) Blood vessel formation: what is its molecular basis? *Cell* **87:**1153-1155.

GERBER, H., VU, T.H., RYAN, A.M., KOWALSKI, J., WERB, Z., and FERRARA, N. (1999) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nature Medicine* **5:** 623-628.

LEACH, R.M. and NESHEIM, M.C. (1965) Nutritional, genetic, and morphological studies of an abnormal cartilage formation in young chickens. *Journal of Nutrition* **86:** 236-244.

LEDWABA, M.F. and ROBERSON, K. D. (2003) Effectiveness of twenty-five-hydroxycholecalciferol in the prevention of tibial dyschondroplasia in Ross cockerels depends on dietary calcium level. *Poultry Science* **82:** 1769-1777.

MAES, C., CARMELIET, P., MOERMANS, K., STOCKMANS, I., SMETS, N., COLLEN, D., BOUILLON, R., and CARMELIET, G. (2000) Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Mechanisms of Development*.**111:** 61-73.

MARILKOVSKY, M. (2002) Thiram inhibits angiogenesis and slows the development of experimental tumors in mice. *British Journal of Cancer,* **86**: 779-787.

MITCHELL, R.D., EDWARDS, H.M. JR, MCDANIEL, G.R., and ROWLAND, G. N. (1997) Dietary 1, 25-dihydroxycholecalciferol has variable effects on the incidences of leg abnormalities, plasma vitamin D metabolites, and vitamin D receptors in chickens divergently selected for tibial dyschondroplasia. *Poultry Science* **76:** 338-345.

NATIONAL RESEARCH COUNCIL (1994) Nutrient Requirements of Poultry.9th edition (rev). National Academy Press, Washington D.C.

NILSSON, O., MARINO, R., DE LUCA, F., PHILLIP, M., and BARON, J. (2005) Endocrine regulation of the growth plate. *Hormone Research* **64:** 157-165.

PINES, M., HASDAI, A., and MONSONEGO-ORNAN, E. (2005) Tibial dyschondroplasia- tools, new insight, and future prospects. *World's Poultry Science Journal* **61:** 285-297.

PINES, M. and HUROWITZ, S. (1991) The role of the growth plate in longitudinal bone growth. *Poultry Science* **70:**1806-1814.

PROVOT, S. and SCHIPANI, E. (2005) Molecular mechanisms of endochondral bone development.
Biochemical and Biophysical Research Communications **328**: 658-665.

RATH, N.C., HUFF, W.E., BAYYARI, G.R., and BALOG, J.M. (1998) Cell death in avian tibial dyschondroplasia. *Avian Disease* **42**: 72-79.

RATH, N.C., HUFF, W.E., BALOG, J.M., and HUFF, G. R. (2004). Comparative efficacy of different dithiocarbamates to induce tibial dyschondroplasia in poultry. *Poultry Science* **83:** 266-274.

RATH, N.C., RICHARDS, M.P., HUFF, W.E., HUFF, G.R., and BALOG, J.M. (2005) Changes in the tibial growth plates of chickens with thiram-induced dyschondroplasia. *Journal of Comparative Pathology* **14:** 41-52.

RATH, N. C., KANNAN, L, PILLAI, P. B., HUFF, W. E., HUFF, G. R., HORST, R. L, EMMERT, J. (2006). Evaluation of the efficacy of vitamin D3 or its metabolites on thiram-induced tibial dyschondroplasia in chickens (Submitted)

RENNIE, J.S. and WHITEHEAD, C.C. (1996) Effectiveness of dietary 25-and 1-hydroxycholecalciferol in combating tibial dyschondroplasia inbroiler chickens. *British Poultry Science* **37:** 413-421.

SAS INSTITUTE, (1994) SAS/STAT User's Guide: (1994) Edition. SAS Institute Inc., Cary, N.C.

US EPA, (2001) Thiocarbamates: A determination of the existence of a common mechanisms of toxicity and a screening level cumulative food risk assessment. http://www.epa.gov/pesticides/cumulative/thiocarb.pdf

VARGAS, M.I., LAMAS, J.M., and ALVARENGA, V. (1983) Tibial dyschondroplasia in growing chickens experimentally intoxicated with tetramethylthiuram disulfide. *Poultry Science* **62:** 1195-1200.

WHITEHEAD, C.C. (1995) The role of vitamin D metabolites in the prevention of tibial dyschondroplasia. *Animal Feed Science and Technology* **53:** 205-210.

WHITEHEAD, C. C., MCCORMACK, H.A., MCTEIR, L., and FLEMING, R.H. (2004) High vitamin D3 requirements in broilers for bone quality and prevention of tibial dyschondroplasia and interactions with dietary calcium, available phosphorus and vitamin A. *British Poultry Science* **45:** 425-436.