Original Article

Effect of thiram on avian growth plate chondrocytes in culture

Komal Singh Rasaputra^{1,2}, Rohana Liyanage³, Jackson O. Lay, Jr.³, Michael F. Slavik²
and Narayan C. Rath¹

¹Agricultural Research Service/ USDA, Poultry Science Center, University of Arkansas, Fayetteville, AR 72701

²Department of Poultry Science, University of Arkansas, Fayetteville, AR 72701

³State Wide Mass Spectrometry Facility, University of Arkansas, Fayetteville, AR 72701

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ABSTRACT — Thiram is a dithiocarbamate pesticide that causes tibial dyschondroplasia (TD), a growth plate defect, in poultry. Deaths of transitional zone chondrocytes appear to interrupt endochondral bone development leading to the broadening of growth plate. The mechanism of action of thiram on chondrocytes is not well understood. Since proteins play major roles in different aspects of cell's metabolism, growth, and survival, the objective of this study was to find whether thiram produces proteomic changes that could impair the development of chondrocytes. The chondrocytes, isolated from proximal tibial growth plates, were cultured with or without a sub-lethal concentration of thiram for 48 hr, and the cell proteins were extracted, and subjected to 2-D gel electrophoresis. The gel images were compared and statistically evaluated using Melanie software to identify differentially expressed protein spots. Of a total of 72 identifiable spots 3 were down-regulated and 2 up-regulated in thiram treated chondrocytes. In-gel trypsin digestion of the protein spots followed by their characterization by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry identified 25 spots comprising of 23 proteins. Two of 3 down-regulated proteins were identified as a heat shock protein 70 (HSP 70) and a GALE (UDP-galactose-4 epimerase) protein isoform I. The up-regulated proteins were Serpin H1, a protein involved in collagen metabolism and a redox sensor NmrA-like (NMRAL) family domain protein-1. Both GALE and NMRAL proteins are implicated in energy metabolism and redox regulation whereas the HSP 70 protects cells against stress, and implicated in chondrocyte hypertrophy, an important event in endochondral bone formation. The failure of chondrocyte protective mechanisms such as associated with protection against cellular stress and energy metabolism appear to be the likely cause for chondrocyte death induced by thiram.

Key words: Thiram, Chondrocyte, Proteins, Chicken, Cell death

INTRODUCTION

Thiram (tetramethyl thiuram disulfide) is a general use dithiocarbamate (DTC) pesticide and an agricultural fungicide (E.P.A., 2001). Embryonic exposure to thiram and similar other DTC induce a variety of skeletal, and craniofacial abnormalities (Van Leeuwen *et al.*, 1990; Robens 1969; van Bortel *et al.*, 2010; Teraoka *et al.*, 2006; Suzuki *et al.*, 2000; Tilton *et al.*, 2006; Haendel *et al.*, 2004). In post hatch poultry thiram produces tibial dyschondroplasia (TD), a common leg problem, where the proximal growth plate cartilage of tibia and tibiotarsal bones fail to undergo

endochondral bone formation (Vargas *et al.*, 1983; Rath *et al.*, 2007; Leach and Monsonego-Ornan, 2007; Pines *et al.*, 2005; Simsa *et al.*, 2007; Orth and Cook, 1994; Farquharson, 2002). TD is characterized by a disproportionate broadening of growth plate due to the retention of nonviable cartilage that fails to resorb and replaced by bone (Rath *et al.*, 1994b, 2005). The dyschondroplastic cartilage is laden with dead or dying chondrocytes (Praul *et al.*, 1997; Rath *et al.*, 1998, 2005). *In vitro*, thiram exhibits cytotoxic effects in nano- to micro molar concentrations depending upon the cell types and densities (Rath *et al.*, 1995; Cereser *et al.*, 2001; Hodgson and Lee, 1977;

Correspondence: Narayan C. Rath (E-mail: narayan.rath@ars.usda.gov)

Rath *et al.*, 2011). However, the early modes of action of thiram that interfere with chondrocyte metabolism and development leading to their death are not well understood. Since proteins constitute both structural and functional infrastructures of tissues, their changes can have adverse biological consequences affecting growth, differentiation, survival, and signaling mechanisms of the cells. Thus, a major goal of toxicoproteomics has been to characterize biochemical changes that lead to disease processes that can be intervened (Kennedy, 2002; Merrick and Witzmann, 2009; Usami *et al.*, 2009). The objective of this study was to find how thiram affects chondrocyte proteome that may explain their premature death leading to the development of TD. Using a pre-determined sublethal concentration of thiram, we report the proteomic changes in chondrocytes in culture that likely interfere with their development and differentiation consequently, the pathogenesis of TD.

MATERIALS AND METHODS

Reagents and chemicals

The following materials, zoom strips pH 3-10, isoelectric focusing (IEF) rehydration buffer (Invitrogen, Carlsbad, CA, USA), recombinant trypsin, lactate dehydrogenase (LDH) CytoTox-One kit (Promega, Madison, WI, USA), C18 OMIX® tips (Varian, Santa Clara, CA, USA), peptide calibration standard (Bruker Daltonics, Germany), Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL, USA), pre-stained protein molecular weight markers (Fermentas, Hanover, MD, USA), Coomassie® Plus protein assay reagent (Pierce, Rockford, IL, USA), culture plates (Costar, Cambridge, MA, USA), 2D clean up kit (GE healthcare, Piscataway, NJ, USA), DMEM/F12 50/50 mix culture medium with L-glutamine (Mediatech Inc., Herndon, VA, USA), collagenase D (Boehringer Mannheim, Indianapolis, IN, USA), ovine testicular hyaluronidase (ICN, Irvine, CA, USA), and fetal bovine serum (FBS) (Valley Biomedical Inc., PA, USA), were purchased from their respective vendors. All other chemicals including thiram (tetramethyl thiuram disulfide), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), antibiotics antimycotic solution, gentamicin, DMSO (dimethyl sulfoxide), ethylenediaminetetraacetic acid (EDTA), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Histopaque-1077, 2, 5-dihydroxybenzoic acid (DHB), and HCCA (α -cyano-4-hydroxycinnamic acid) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

The animal experiments were approved by the University of Arkansas Institutional Animal Use and Care committee.

Chondrocyte isolation and culture

Chondrocytes were isolated from the proximal tibial growth plate cartilage of 2-wk old chickens following an earlier procedure (Rath *et al.*, 1994a, 1995). Chickens were killed and the growth plates were exposed by snapping up of articular cartilage followed by the harvest of cartilage tissues with the help of a 5 mm curette under sterile conditions. Pooled cartilage tissues from 6 chickens were washed in DMEM/F-12 medium containing 10 mM HEPES, antibiotic, antimycotic solution then digested overnight in the same medium supplemented with 10% FBS, and 1mg/ml each of collagenase-D, and ovine testicular hyaluronidase at 37°C for 18-20 hr in a cell culture incubator to release chondrocytes. After removing the fast settling debris, the cells in the supernatant were layered over a Histopaque-1077 density gradient medium and centrifuged at 400 g for 30 min. The cells at the Histopaque and culture medium interface were washed twice with serum free DMEM/F-12 then reconstituted in the media containing 10% FBS. The cells were seeded in 12-well plates at concentrations of 10^6 or 5×10^6 cells/ml per well and cultured overnight for establishment. All experiments were done in triplicate. On the following day, the supernatant media were carefully removed and replaced with fresh medium containing thiram or an appropriate control medium for a period of 48 hr. Thiram was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 0.1 M from which subsequent dilutions were made using culture media. Preliminary experiments were done to determine a sub-lethal concentration of thiram for proteomic assay using Cytotox one lactate dehydrogenase (LDH) assay kit measuring LDH release into the culture medium as the indicator of the loss of membrane integrity and cell death (Vanderlinde, 1985). The morphology of the control and thiram treated chondrocytes were examined, and photographed using an Olympus IX 70 microscope fitted with a CoolSnap-Pro camera (Media cybernetics, Silver Spring, MD, USA).

Based on the LDH release results 1 μ M thiram was determined to be sub lethal to chondrocytes cultured at the concentration of 5×10^6 cells/ml thus, chosen to evaluate its effects on proteome following 48 hr of treatment.

Protein extraction and two dimensional gel electrophoresis (2DGE)

Replicate cultures of chondrocytes (n = 3 per group) were gently washed thrice with 1 ml of Hank's basal

salt solution (HBSS) each and the cellular proteins were extracted using procedures described earlier (Belluoccio *et al.*, 2006; Rasaputra *et al.*, 2010). The cell layer was lysed and extracted with 0.5 ml of 4 M guanidine hydrochloride (GuHCl) containing 50 mM sodium acetate pH 5.8, 10 mM EDTA, 65 mM dithiothreitol (DTT), and 1X concentration of Halt proteinase inhibitors at 4°C for 20 hr. The insoluble cell debris was removed by centrifugation at 12,000 g for 10 min at 4°C and the proteins in the supernatant precipitated with a 2-D cleanup solution per manufacturer's instructions before isoelectric focusing (IEF) and 2D gel electrophoresis (2DGE). The protein pellet was solubilized in 200 µl of IEF rehydration buffer consisting of 8 M urea, 2% CHAPS, 2% immobilized pH gradient (IPG) buffer, and 0.001% bromophenol blue. The concentration of protein in the solutions was determined by Coomassie Plus protein assay reagent with bovine serum albumin (BSA) as the standard made with the rehydration buffer. The protein extracts of the control and treated cells were equalized using blank buffer before 2D gel electrophoresis.

Isoelectric focusing was done with 20 µg of protein in 155 µl of rehydration buffer containing 65 mM dithiothreitol (DTT) using three separate extracts in each group. The samples were loaded onto a linear 7 cm IEF strip, pH 3-10, for first dimension separation using a Zoom IPG Runner mini-cell (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The proteins focused on IPG strips were first reduced with 2% DTT then alkylated with 2.5% iodoacetamide in a solution containing 50 mM Tris-HCl, 2% SDS, 30% glycerol, and 6 M urea for 15 min each. The second dimension separation was done using electrophoresis on 12% polyacrylamide gels in a Multicell Protean II BioRad apparatus (Richmond, CA, USA) with 12 mA constant current per gel. A protein molecular weight (MW) marker was used as reference. The gels were stained with Coomassie blue, scanned, photographed using Gel logic 2200 imaging system (Carestream Health, CT, USA), and the 2D images were analyzed by Melanie 5.0 software (Swiss Institute of Bioinformatics, Geneva, Switzerland) to determine differentially expressed protein spots. The intensities of individual spots were calculated using feature detection algorithm with one control gel as reference to which the spots in all other gels were aligned, matched, and a number was assigned to each spot for pairing. Mismatched spots were corrected manually and the relative intensities of each spot were calculated with respect to the sum of the intensities of all spots in the gel under consideration. Student's t-test was performed using average intensity of a spot from each group thus, identifying the differentially

expressed proteins with respect to control group considering a *p* value of ≤ 0.05 as significant.

Trypsin digestion and identification of proteins

The Coomassie stained protein spots were excised, destained with 50% acetonitrile (ACN), and the gel pieces were dried using a Speedvac concentrator centrifuge. The dried gel pieces were incubated with 20 ng of trypsin contained in 25 mM ammonium bicarbonate, enough in volume, to hydrate, and digest the protein in the gel. After an overnight digestion, the tryptic peptides were eluted from the gel matrix with 60% ACN containing 5% formic acid and the total volume reduced to ~10 µl using a Speedvac centrifuge followed by desalting with C18 OMIX® tips per the manufacturer's protocol. The tryptic peptides were eluted with 60% ACN containing 0.1% trifluoroacetic acid (TFA) from which a 2 µl aliquot was mixed with an equal volume of saturated α -cyano 4-hydroxycinnamic acid (HCCA) and spotted on a Bruker MTP 384 stainless steel MALDI target plate. The tryptic peptide mass fingerprint (PMF) and the MALDI LIFT-TOF/TOF (MS/MS) data were obtained using an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) in positive ion mode. The combined PMF and MS/MS data were searched using MASCOT search engine interfaced with Bruker Biotoool version 3.1 software to identify the proteins. The NCBI database was searched under chordata taxonomy. Monoisotopic peptide mass tolerance of ± 200 ppm, MS/MS tolerance of ± 0.8 Da, one missed cleavage, fixed carboxyamidomethylation of cysteines, and variable methionine oxidation were used during the data base search. All the identifications were based on a match of at least 2 peptides and a combined MASCOT score (MS and MS/MS) > 70 with $p \leq 0.05$.

RESULTS

LDH release and cell morphology

Figures 1 and 2 represent the relative LDH activities in the cell culture supernatant and the image of cell layers of the after 48 hr of treatment with thiram. Chondrocytes treated with 1 µM thiram showed no significant change in LDH release into the culture medium compared with the controls indicating that the cells remained viable at this concentration (Fig. 1). Thiram nonetheless, caused progressive deterioration of cell morphology indicated by loss of cell border clarity and hypotrophic change that was clearly evident at 10 µM concentration (Fig. 2c) that significantly increased the release of LDH into the media ($p \leq 0.05$). In low density culture (10⁶ cells/ml) thiram at the concentration of 1 µM, produced significant LDH release

into the medium indicating cytotoxic effects (not shown).

Protein analysis

Seventy-two spots were matched among six 2D gel images of control and the thiram treated chondrocyte proteins. Densitometric comparisons of the matched spots showed quantitative changes in 5 spots with $p \leq 0.05$ of which 3 were down-regulated in thiram treated chondrocytes with only two identified as heat shock protein 70 (HSP 70) and UDP-galactose 4-epimerase (GALE) protein, respectively (Fig. 3). The up-regulated proteins in thiram treated chondrocytes were identified as a nmrA-like (NMRAL) family domain containing protein-1 and Serine protease inhibitor H1 (SerpH1) precursor protein. The MALDI-TOF peptide mass fingerprints (PMF) of the HSP70 spot are shown in Fig. 4 with m/z peaks assigned to appropriate tryptic peptide sequences. Domains of the sequences of HSP 70 are confirmed by MS/MS. Only 25 spots consisting of 23 proteins could

be identified on data base search from the total 72 spots (Fig. 3 and Table 1).

DISCUSSION

Acute or chronic exposures to pesticides pose risks of health hazards (Maroni *et al.*, 2000; Damalas and Eleftherohorinos, 2011). One of the major mechanisms through which the toxic chemicals induce pathologies is by disrupting vital metabolic processes which include interference of protein functions (Smith, 2001). Both qualitative and quantitative changes of essential proteins can limit their functions by compromising their roles in various aspects cell's metabolism such as, signal transduction mechanisms, enzymatic functions, interactions with other proteins and biochemical that can lead to the disease processes (Wetmore and Merrick, 2004). Many environmental chemicals are tested for their developmental toxicities and teratogenicity using skeletal malformations (Carney and Kimmel, 2007). Although proteomic changes conceivably underlie various skeletal malformation yet studying skeletal cell proteomics with respect to toxic substances has been limited. The preponderance of extracellular matrix (ECM) molecules such as collagens and highly anionic and nonhomogeneous proteoglycans which interfere with analytical procedures involving isoelectric focusing and 2D gel electrophoresis (Belluoccio *et al.*, 2006; Wilson *et al.*, 2008; Ruiz-Romero and Blanco, 2009; Gharbi *et al.*, 2011). The cell culture system circumvents some of these problems by taking advantage of the fact that the ECM proteins are secreted into the culture media and those retained in the cell layer are much lower in concentration compared with *in vivo* obtained tissue proteins to interfere with 2D gel electrophoresis.

Thiram exhibits cytotoxicity to chondrocytes and other cells (Rath *et al.*, 1995; Cereser *et al.*, 2001). The tox-

Fig. 1. Lactate dehydrogenase (LDH) activity of chondrocyte culture media at 48 hr following treatment with or without thiram * indicates $p \leq 0.05$.

Fig. 2. Bright field image of chondrocytes at 48 hr after treatment with or without thiram (200 X) a. 0 μM thiram, b. 1 μM thiram, c. 10 μM thiram. Thiram at 10 μM concentration shows damaging effects indicated by loss of cellular clarity and hypotrophic changes.

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Fig. 3. 2D gel profiles of chondrocyte protein extracts stained with Coomassie blue R-250. The regions in the rectangular box highlight the protein spots from the identical areas of control and thiram treated chondrocyte extracts separated on 2D gels showing, a. Heat shock protein 70 (HSP 70), b. Serpin H1 precursor, c. Predicted: similar to GALE protein isoform and d. NMRA-like family domain-containing protein 1. MW kDa - molecular weight in kilo-Daltons.

Fig. 4. MALDI-tryptic peptide mass fingerprint of the gel spot identified as HSP-70, a protein downregulated in thiram treated chondrocytes and identified by MASCOT data base search. Figure shows m/z peaks that matched to the tryptic fragments of HSP-70 sequence. Sequence regions shown in blue are confirmed by MALDI LIFT-TOF/TOF (MS/MS).

icity appears to be related to the density of the cells. In sub-lethal concentration thiram although did not produce chondrocyte death at 48 hr, it caused changes in proteins that appear to be essential for their survival in long

term basis. An NmrA-like (NMRAL) family domain protein 1 was up-regulated by thiram which is a redox sensor protein induced upon depletion of NAD(P)H dependent enzymes (Zhao *et al.*, 2008; Kavanagh *et al.*, 2008). NAD(P)H dependent enzymes are oxidoreductases, short-chain dehydrogenase/ reductase, enzymes that are essential for carbohydrate, lipid, amino acid metabolism, and for the maintenance of cellular homeostasis necessary for survival and stress response (Kensler *et al.*, 2007; Kavanagh *et al.*, 2008). Although the effects of thiram on different oxidoreductase enzymes are unknown, it has been reported to inhibit aldehyde dehydrogenase (ALDH), 11 β hydroxy steroid dehydrogenase, and glutathione reductase which are essential in cellular functions (Sanny and Weiner, 1987; Staub *et al.*, 1995; Elskens and Penninckx, 1997; Cereser *et al.*, 2001; Atanasov *et al.*, 2003). The ALDH inhibitory effect of disulfiram, the ethyl analog of thiram, is the basis of its use in alcohol aversion therapy (Petersen, 1992). The ALDH gene superfamily of enzymes also play important role in protection against cellular stress (Jackson *et al.*, 2011; Kotchoni *et al.*, 2010). In the event of deficiencies of these enzymes induced by toxicants, the cells are likely to experience stress. The inability to recover from cellular stress may lead to their death. Another protein, up-regulated in thiram treated chondrocytes, was Serpin H1 precursor protein, alternatively known as heat shock protein 47 (HSP47). HSP47

Table 1. Chicken chondrocyte proteins identified by MALDI-TOF-MS and MALDI LIFT-TOF/TOF MS/MS.

Spot #	Protein	Accession #	PMF MS/MS Score	No. of peptides matched
1	Unnamed protein product	gi 74220199	74	7
2	Actin prepeptide	gi 178067	124	2
3	Predicted: ADP-ribosylation factor guanine nucleotide factor 6 isoform 4	gi 114619022	79	6
4-7	Collagen alpha-1(II) chain	gi 45383309	114	16
*8	Heat shock protein-70	gi 45544521	278	11
9	Unnamed protein product	gi 428	211	3
10	Serum albumin	gi 76445989	210	9
11	Alpha-1-antiproteinase precursor	gi 27806941	75	2
12	Prolyl-4-hydroxylase	gi 63739	187	5
13	Vimentin	gi 114326309	148	13
14	Prolyl-4-hydroxylase	gi 63739	143	4
15	UDP-glucose 6-dehydrogenase	gi 60302808	113	6
16	Cartilage matrix protein precursor	gi 71896317	162	10
17	Cartilage matrix protein precursor	gi 71896317	104	17
18	Predicted: similar to laminin receptor 1 isoform 1	gi 73949345	119	5
19	Predicted: hypothetical protein XP_533132	gi 73964667	325	15
20	alpha-enolase	gi 46048768	200	5
*21	Predicted: similar to GALE protein isoform 1	gi 118101799	126	3
**22	Serpin H1 precursor	gi 45384240	111	6
23	Predicted: endoplasmic reticulum protein 29	gi 224071623	117	4
**24	NmrA-like (NMRAL) family domain-containing protein 1	gi 71897147	127	8
25	Triosephosphate isomerase	gi 34221912	73	2

* Correspond to differentially down-regulated or ** up-regulated chondrocyte proteins by treatment with thiram.

is a collagen binding molecular chaperon found in collagen secreting cells such as chondrocytes that is elevated under stress (Hart *et al.*, 2000; Natsume *et al.*, 1994). The absence of HSP 47 causes embryonic death and severe chondrodysplasia (Nagata and Hosokawa, 1996; Masago *et al.*, 2012). Serpin H1 is also up-regulated in rheumatoid arthritis (Maciejewska-Rodrigues *et al.*, 2010). The up-regulation of Serpin H1 in chondrocytes further suggests the stress response of chondrocytes to thiram. However, another stress associated protein, HSP70, was significantly down regulated in thiram treated chondrocytes. HSP70 protects cells against stress (Mosser *et al.*, 2000; Guzhova and Margulis, 2006). It is strongly expressed in hypertrophic chondrocytes and prevents the production of apoptotic proteins (Otsuka *et al.*, 1996; Vanmuylder *et al.*, 1997). HSP 70 also confers chondroprotective effects in experimentally induced arthritis (Etienne *et al.*, 2008). The thiram treated chondrocytes showed consider-

able decreases in size. The reciprocal relationship of HSP 70 and HSP 47 in thiram treated chondrocytes however, is not clear. But a decrease in HSP 70 levels in the event of elevated cellular stress can prevent recovery leading to cell death. Genin *et al.* (2008) showed chondrocytes of hypertrophic zone of TD-affected growth plate cartilage synthesizing HSP 70 which they suggest was to be related to hypoxia stress. The discrepancy between our results can be due the techniques of analysis. Whereas Genin *et al.* (2008) used immunohistochemical localization of HSP 70 in TD-affected growth plates showing its up-regulation in surviving hypertrophic chondrocytes, our results were obtained with the chondrocytes that were uniformly affected with thiram, and have metabolically failed. Ribeiro *et al.* (2004) similarly, observed a reduction in the level of HSP70 proteins in TD-affected cartilage of broilers with severe lesions. Another identified protein named UDP-galactose 4-epimerase (GALE) was also down-reg-

ulated in thiram treated chondrocytes. GALE protein is an enzyme responsible for the epimerization of UDP-glucose and UDP-N-acetyl glucosamine to UDP-galactose, and UDP-N-acetyl galactosamine, and vice versa (Niou *et al.*, 2009; Bang *et al.*, 2009). GALE plays crucial role in energy metabolism through production of UDP-glucose and UDP- N-acetyl glucosamine necessary for mucopolysaccharide, glycoprotein, glycolipid, and the proteoglycan biosynthesis (Sweeney *et al.*, 1993; Lai *et al.*, 2009).

The mechanism involved in the pathogenesis of naturally occurring TD is poorly understood. Endoplasmic reticulum (ER) stress is implicated in cellular apoptosis (Walter and Ron, 2011). ER stress in hypertrophic chondrocytes causes a variety of cartilage pathology including chondrodysplasia (Rajpar *et al.*, 2009). Leach and Monsonego-Ornan (Leach and Monsonego-Ornan, 2007) hypothesized ER stress as a mechanism for the pathogenesis of TD. Whether thiram induces ER stress in chondrocytes is unknown but our previous results along with the current study shows that an induction of cellular stress perhaps compromises energy metabolism of the chondrocytes, affects their secretory function, and arrests signal transduction mechanisms that may be consequential to their subsequent death (Rath *et al.*, 2005; Rasaputra *et al.*, 2010). The hypertrophic changes in transition zone chondrocytes of growth plate most likely facilitate the incorporation of thiram into those cells leading to their premature death.

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