

Muscular contractions in the zebrafish embryo are necessary to reveal thiuram-induced notochord distortions

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Abstract

Dithiocarbamates form a large group of chemicals that have numerous uses in agriculture and medicine. It has been reported that dithiocarbamates, including thiuram (tetramethylthiuram disulfide), cause wavy distortions of the notochord in zebrafish and other fish embryos. In the present study, we investigated the mechanism underlying the toxicity of thiuram in zebrafish embryos. When embryos were exposed to thiuram (2–1000 nM: 0.48–240 µg/L) from 3 h post fertilization (hpf) (30% epiboly) until 24 hpf (Prim-5), all embryos develop wavy notochords, disorganized somites, and have shortened yolk sac extensions. The thiuram response was specific and did not cause growth retardation or mortality at 24 hpf. The thiuram-dependent responses showed the same concentration dependence with a waterborne EC₅₀ values of approximately 7 nM. Morphometric measurements revealed that thiuram does not affect the rate of notochord lengthening. However, the rate of overall body lengthening was significantly reduced in thiuram-exposed animals. Other dithiocarbamates, such as ziram, caused similar malformations to thiuram. While expression of genes involved in somitogenesis was not affected, the levels of notochord-specific transcripts were altered after the onset of malformations. Distortion of the notochord started precisely at 18 hpf, which is concomitant with onset of spontaneous rhythmic trunk contractions. Abolishment of spontaneous contractions using tricaine, α-bungarotoxin, and a paralytic mutant *sofa potato*, resulted in normal notochord morphology in the presence of thiuram. These results indicate that muscle activity is necessary to reveal the underlying functional deficit and suggest that the developmental target of dithiocarbamates impairs trunk plasticity through an unknown mechanism.

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Introduction

Agricultural agents or pesticides applied to farmland pose a possible risk to humans and wildlife due to runoff into rivers, lakes, oceans, or groundwater. Consequently,

pesticides could be a threat to fish and other aquatic organisms. Dithiocarbamates constitute a major class of pesticides and have been widely used for several decades, as they are believed to be relatively safe for mammals. Thiuram or thiram (tetramethylthiuram disulfide) is one of the representative dithiocarbamate pesticides, and has been used as a fungicide on seeds and animal repellent. In addition, thiuram is used as a major vulcanizing agent in rubber manufacturing. Thiuram is one of the dimethyl-

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dithiocarbamates which exert their effects through a number of sulfhydryl (SH) enzymes in microorganisms perhaps through cation chelation (Stromme, 1963). Several lines of evidence indicate that after administration to cells, thiuram is rapidly reduced to its corresponding thiol, dimethyldithiocarbamic acid, which is considered the active moiety because of its chelating properties (Cremllyn, 1979). While thiuram and some other dithiocarbamates have been shown to produce immune responses (reviewed in Pruett et al., 2001), thiuram also shows developmental toxicity in various animals, including mammals. It has been reported that thiuram caused cleft palate, wavy rib formation, distortion of long limb bones, and small lower jaw in mice (Roll, 1971), and fused rib, small lower jaw, and short wavy tail in hamster (Robens, 1969). Thiuram also caused hydrocephaly in mice and rats (Short et al., 1976). In addition to developmental toxicity, genetic toxicity has been reported following thiuram exposure (Agrawal et al., 1997). Furthermore, thiuram could be regarded as a candidate endocrine disrupter, based on a report of testicular atrophy and abnormal spermatogenesis in mice (Mishra et al., 1998).

In adult fish, many cases of vertebral disorders, such as vertebral distortion and fracture, have been reported, and pesticides have been posed as one of the possible causes, because of the widespread use. In experimental exposures, malathion, an organophosphate, caused vertebral fracture in African catfish, possibly due to abnormal trunk muscle contraction caused by inhibition of choline esterase (Lien et al., 1997), or lysyl oxidase, prolyne hydroxylase (Snawder and Chambers, 1993) and lysyl hydroxylase (Samimi and Last, 2001), all of which are indispensable for collagen fiber maturation. Although there are limited dithiocarbamate developmental toxicity studies, it has been reported that rainbow trout embryos showed unique morphological changes, such as wavy notochord, disorganized somites, and shortened yolk sac extension upon exposure to thiuram and other dithiocarbamates (van Leeuwen et al., 1986). When the dithiocarbamate medicine disulfiram was used to inhibit retinal aldehyde dehydrogenase in zebrafish, notochord distortions were unexpectedly produced (Marsh-Armstrong et al., 1995). Similar malformations were produced in disulfiram-exposed flounder embryos (Suzuki et al., 2001). Although the mechanism remains unclear, it was speculated that cationic metal chelation, a common property of dithiocarbamates, such as thiuram, may play a role in the toxic response. Recently, it was reported that sodium metam, a mono methyl dithiocarbamate and its breakdown product methyl isothiocyanate (MITC) causes similar trunk malformation in zebrafish embryos (Haendel et al., 2004).

In order to clarify the mechanism of toxicity produced by dithiocarbamates, we have investigated the effects of thiuram and other dithiocarbamates in zebrafish, which is an outstanding model fish for developmental toxicology studies (Teraoka et al., 2003). Our

results indicate that notochord morphology is a principle target of thiuram, and most importantly, that trunk contractions are absolutely necessary to reveal the notochordal response.

Materials and methods

Zebrafish embryos and chemical exposure. Fertilized eggs were obtained from natural mating of adult zebrafish (AB line) according to the *Zebrafish Book* (Westerfield, 1995). Adult fish and embryos were maintained at 28.5 °C with a lighting schedule of 14 h light and 10 h dark. Embryos were collected within 1 h of spawning, rinsed, and placed into petri dishes. Within 3 h post fertilization (hpf) of spawning, the embryos were exposed to graded concentrations of waterborne thiuram (CAS # 137-26-8: Wako Pure Chemical, Japan). Stock solutions were prepared in 100% dimethyl sulfoxide (DMSO). Appropriate concentration of thiuram was present in 3 mL of Zebrafish Ringer solution (38.7 mM NaCl, 1.0 mM KCl, 1.7 mM HEPES-NaOH pH 7.2, 2.4 mM CaCl₂) in 3.5-cm petri dishes (Asahi Techno, Japan) for the duration of the experiment ($n = 10$ embryos/dish). Final DMSO concentration was 0.1%. Exposure of other dithiocarbamates such as ziram (CAS # 137-30-4: Wako Pure Chemical, Japan) and disulfiram (CAS # 97-77-8: Kanto Kagaku, Japan) was carried out following the same protocol. As mechanically dechorionized embryos with two fine forceps showed the same sensitivity to thiuram and other chemicals in preliminary experiments, all embryos were used with natural chorion. The *sofa potato* mutants were originally identified in a genetic screen in the Nüsslein-Volhard Laboratory, Max-Planck-Institute for Developmental Biology and were kindly provided by Paul Brehm, State University of New York at Stony Brook. Chemicals unless indicated were obtained from Sigma (St. Louis, MO) and Kanto Kagaku.

Morphometry. Live 24-h embryos were observed microscopically in 3% carboxymethyl cellulose sodium salt/Zebrafish Ringer solution after being anesthetized with tricaine (MS222, Sigma). Lateral embryonic images were digitally acquired (Penguin 150 CL, Pixera, Los Gatos, CA) under stereoscopic microscope (SZX12, Olympus, Japan). The length of the total body and notochord in zebrafish embryos was quantified using standard measurement tools in image analysis software (Photoshop 7.0, Adobe). Total body length was determined by drawing and measuring a line that extended from the tip of the head to the end of the caudal fin. The contour length of the notochord was estimated by manual tracing the convoluted notochord with a line in the center of the notochordal cells. The same magnification was used for each image capture and measurement. The units for comparison are expressed as pixels (inset photos, Fig. 3).

Techniques to inhibit spontaneous motion. Control and thiuram-exposed embryos were incubated with 0.04% w/v tricaine (MS222) to completely cancel spontaneous trunk contractions. Tricaine was applied beginning at 17 and continued through 24 hpf and the resulting notochord morphologies were quantified at 24 hpf. Alpha-bungarotoxin (0.125–0.25 mM) was injected into yolk of 10–14 hpf embryos according to Lefebvre et al. (2004). Injected volumes were around 100 pL. The offspring from a heterozygotic spawning pair were exposed to 20 nM thiuram beginning at 3 hpf and continued through 24 hpf. To phenotypically screen the embryos, the frequency of spontaneous bends were calculated over a 5-min period using continuous video analysis. Embryos with no spontaneous bends are the *sop*^{-/-} genotype, and the embryos capable of bends are either *sop*^{+/+} or *sop*^{+/-}.

Morphological techniques. Whole-mount in situ hybridization was carried out according to Barth and Wilson (1995), as described elsewhere (Teraoka et al., 2002). 0.003% 1-phenyl-2-thiourea was included in Zebrafish Ringer solution from 24 hpf to prevent pigmentation of zebrafish larva. Plasmid DNAs for digoxigenin-antisense probe were generous gifts from Dr. S.W. Wilson. The alkaline phosphatase-dependent activity was detected with BM-purple or Fast red (Roche, Switzerland) substrates. After paraformaldehyde fixation, the stained embryos were cleared in 70% glycerol and microscopically observed and imaged. For electron microscopy, the embryos were fixed in a solution of 2% paraformaldehyde and 1% glutaraldehyde for 2 h or overnight at 4 °C as previously described (Imagawa et al., 1991). After dehydration with a series of alcohol, the embryos were embedded in Lowicryl K4M (Chemische Werke Lowi, Germany) at -20 °C. Ultrathin sections were stained with aqueous uranyl acetate prior to microscopic imaging.

Statistics. Results are presented as mean ± SEM. Significant differences between means were determined by one-way ANOVA followed by Scheffe's test ($P < 0.05$). EC₅₀ values were calculated according to Probit's method.

Results

Embryonic responses to thiuram

To evaluate the embryonic response to thiuram, embryos continuously exposed to thiuram from 3 hpf (30% epiboly stage) and gross morphology was characterized at 24 hpf. One of the most striking thiuram-induced changes was the notochord. In embryos treated with thiuram, notochords showed wavy dorso-ventral distortion, while the notochords in non-exposed embryos were consistently straight (Fig. 1). While the severity of the distortion was consistent among treated embryos, the distortions were remarkable only in the mid trunk, but not in the tail region. Thiuram-exposed embryos had disorderly packed, spherical-shaped, notochordal cells, instead of the regularly disk shaped cell in the controls (Figs. 1B, E). The myotomes of thiuram-exposed embryos do not have the typical chevron V-shape (Fig. 1C compared to Fig. 1F). In addition, the yolk sac extensions of treated embryos were consistently shorter and thicker compared to those of controls (Fig. 1A compared to Fig. 1D).

In order to determine the concentration–response relationship of several morphological changes and mortality, embryos were continuously exposed with several waterborne concentrations of thiuram up to 20 nM (Fig. 2) between 3 and 24 hpf. The percent incidence of each thiuram-dependent endpoint was determined at 24 hpf.

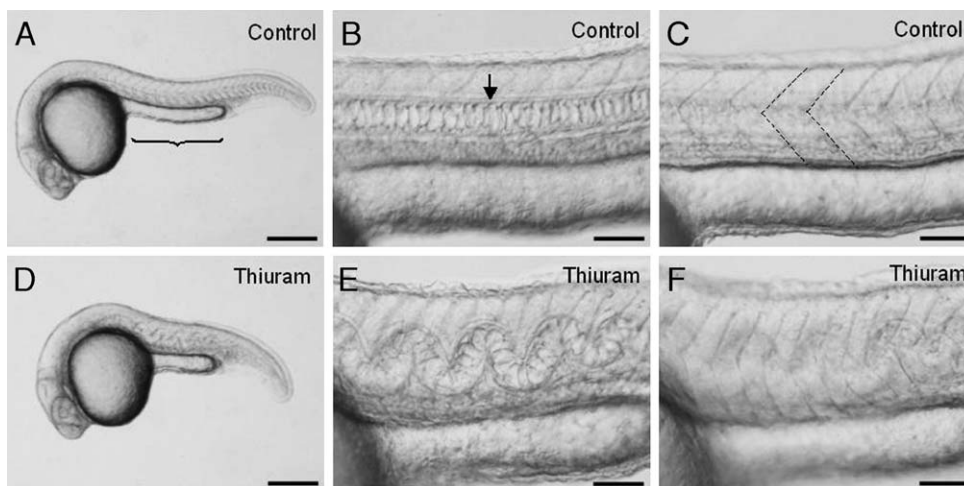


Fig. 1. Morphological disorganizations of zebrafish embryos by thiuram. Embryos exposed to thiuram (D–F: 20 nM) and non-treated control embryos (A–C) from 3 hpf until observation at 24 hpf. All lateral images of live embryos. (A, D) Whole body; (B, E) focused on notochord in the trunk; (C, F) focused on somites in the trunk. Curly bracket in panel A and arrow in panel B indicate yolk sac extension and notochord, respectively. A normal somite was highlighted with dashed lines in panel C. Scale bars in panels A and D: 300 μm; scale bars in panels B, C, E, and F: 100 μm.

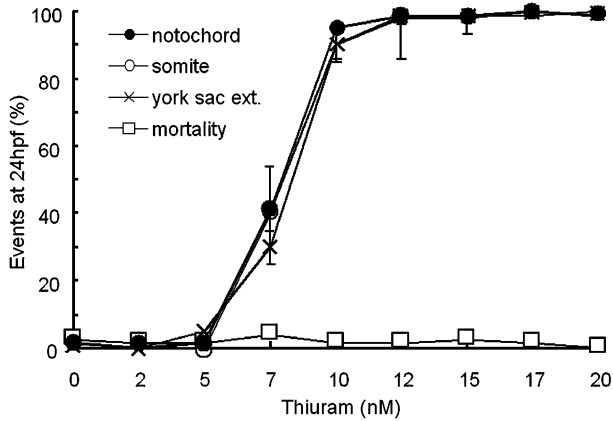


Fig. 2. The same concentration dependency of various trunk malformations by thiuram. Percentages of embryos with wavy notochord (closed circles), disorganized somites (open circles), shortening of yolk sac extension (crosses), and mortality (open squares) were determined at 24 hpf. Each percentage was estimated with 10 embryos and the experiment was repeated 6 times. Symbols and associated vertical line are mean \pm SEM ($n = 6$: 60 embryos/treatment in sum).

Mortality did not occur in any of the exposure groups. Concentrations as high as 1000 nM did not lead to 24-h embryonic mortality (data not shown). For each of the identified endpoints, notochord distortion, shortening of yolk sac extension, and somite disorganization, the percent incidences versus concentration were the same. The estimated EC_{50} values were approximately 7 nM and minimal concentrations to produce a 100% response were between 10 and 20 nM.

Developmental notochord and body lengthening

To determine the rate of body and notochord lengthening during developmental progression, we took advantage of the transparency of the zebrafish embryo and acquired digital images every 30 min between 17 and 24 hpf from control, and embryos exposed to 20 nM thiuram. In zebrafish embryos, two somites are formed per hour from 10 hpf (tail bud) to 24 hpf. From the acquired images, the total body length and notochord lengths were estimated at each time point. Importantly, 20 nM thiuram did not affect the rate of somite formation suggesting that this dithiocarbamate does not globally delay development. As indicated in Fig. 3A, the rate of body lengthening in thiuram-exposed embryos was lower than that in control embryos, beginning at 20 hpf. The body lengths became more disparate with further embryonic development. On the other hand, when notochord lengths were estimated, the average lengths in thiuram-exposed and control embryos were nearly identical at each measured time point (Fig. 3B). These results indicate that the wavy notochord may result from a decreased rate in trunk lengthening without a change in notochord growth.

Although data are not presented, we also studied the effects of the other dimethyl-dithiocarbamates including the pesticide ziram and *N*-methyl-dithiocarbamate (disulfiram). Disulfiram is not a pesticide, but instead a medication commonly used to treat alcoholism by inhibiting acetaldehyde metabolism. Both ziram and disulfiram produced exactly the same phenotype as thiuram, including wavy

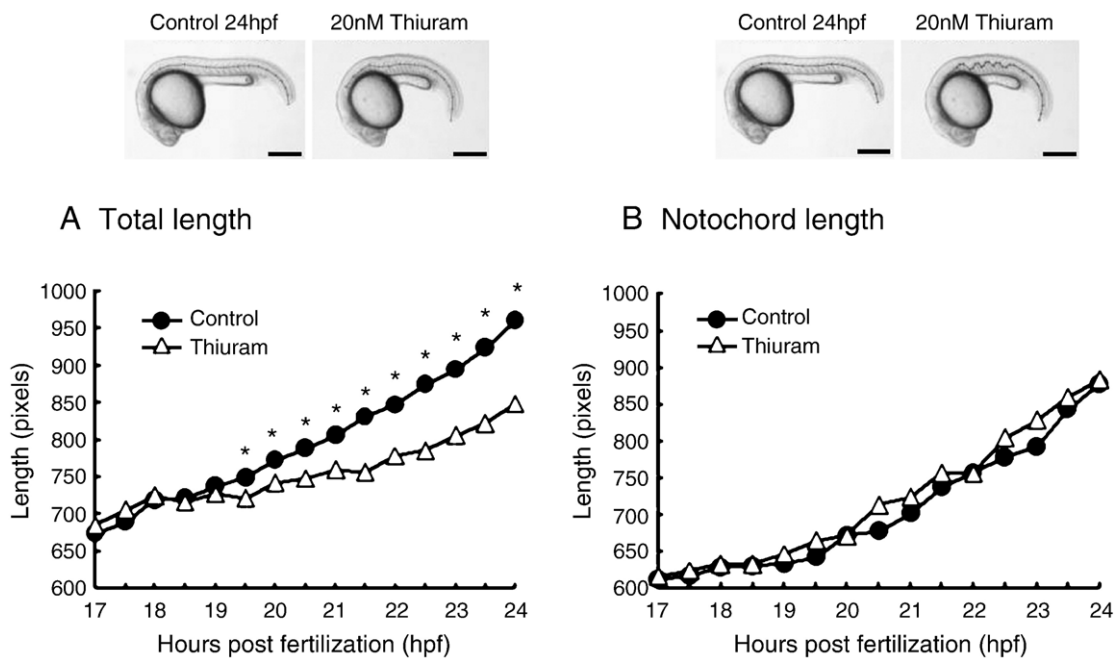


Fig. 3. Thiuram exposure does not impact notochord growth. Digital images in lateral view were captured for control (closed circles) and thiuram-exposed (20 nM: open triangles) embryos from 16 hpf until 30 hpf. Using imaging software (inset photos), total body length (A) and notochord length (B) were measured. Each symbol line is mean of 10 embryos/group. Vertical line for SEM cannot be visible as they are hidden by symbols. * $P < 0.05$. Scale bars in inset photos: 300 μ m.

notochord development, disorganized somites, and reduced yolk sac extensions. The EC_{50} values were 26 nM for ziram and 16 nM for disulfiram. Minimal concentrations for maximal occurrence (100%) were about 50 nM for ziram and 30 nM for disulfiram. These results strongly suggest that the underlying mechanism of action for dithiocarbamates is similar.

Time course of distortion of notochord and somites

In order to more precisely determine the developmental time point at which the notochord first becomes distorted, we continuously observed, and digitally imaged, thiuram-exposed embryos between the 16 to 30 somite stages (17 to 24 hpf). The notochord morphology of embryos exposed to 20 nM thiuram beginning at 3 hpf, was indistinguishable from controls until 17 hpf. The notochords appeared straight and the muscle myotomes were arranged normally (data not shown). With continuous observation, the distortions first became evident at 18 hpf, and complete wave morphology was formed within 63 min (Figs. 4A–F). Surprisingly, the myotomes, at the gross level, appeared to form normally until 17 hpf, after that, the typical chevron shape is lost, and instead they appear crooked, U shaped, and without sharp divisions. During these imaging time course studies, we made the observation that wavy notochord became evident soon after the embryo imitated spontaneous back and forth contractions. The rhythmical spontaneous contractions are the earliest behaviors in zebrafish, originate from spinal neuron innervation, and are independent of higher brain inputs (Saint-Amant and Drapeau, 1998). Importantly, initiation of the spontaneous behavior was the same in control and thiuram-exposed embryos, indicating that thiuram did not cause a developmental delay. Furthermore, these results indicate that the trunk musculature is normal and its innervation has not been impacted by thiuram.

Spontaneous trunk contractions are necessary to reveal dithiocarbamate-induced notochordal response

With the concomitant development of spontaneous motion and the notochord distortions, it became clear that the spontaneous contractions may be involved, or induce, the notochord deformity. To test this hypothesis, we used three different approaches to inhibit early embryonic spontaneous behavior. If movement were necessary to induce the notochord distortion in thiuram-exposed animals, paralyzed, thiuram-exposed embryos should have notochords with normal morphology. The common fish anesthetic tricaine (MS222) that targets sodium channels was used at 0.04% to completely cancel spontaneous trunk movements. Tricaine was applied to control and thiuram-exposed embryos beginning at 17 and continued through 24 hpf. In embryos exposed to 20 nM thiuram, the expected notochord distortions are produced compared to control (compare Figs. 5A and B). In the presence of tricaine, the thiuram-induced notochord distortion was completely abolished (Fig. 5C: 20 of 20 embryos). Since tricaine is not a selective antagonist for sodium channel, which is necessary for cholinergic transmission in the endplate, it is possible that tricaine blocked the notochord distortion independent of its impact on contraction. α -Bungarotoxin, a snake venom-derived toxin that selectively inhibits acetylcholine receptors, was used to block spontaneous contractions. Injection of α -bungarotoxin into embryos has been shown to completely abolish spontaneous motion (Lefebvre et al., 2004). When α -bungarotoxin was injected into embryos, most embryos are markedly paralyzed. In the presence of thiuram, the paralyzed animals had notochords with normal morphology (Fig. 5D: 30 of 37 embryos). Finally, we have taken advantage of the available paralytic mutant *sofa potato* (*sop*) which lacks functional acetylcholine receptors (Ono et al., 2004). When heterozygotic pairs of *sop* adults

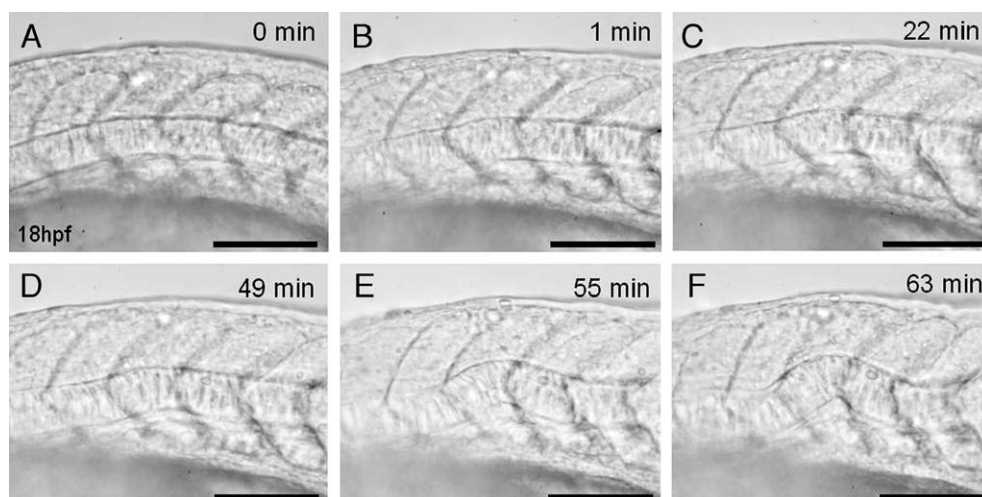


Fig. 4. Time course analysis to capture onset of thiuram-induced trunk distortion. Trunk region of thiuram-exposed embryos (20 nM) were continuously observed using a stereo microscope beginning at 18 hpf. Single-frame images were captured at the indicated times points. Scale bars: 100 μ m.

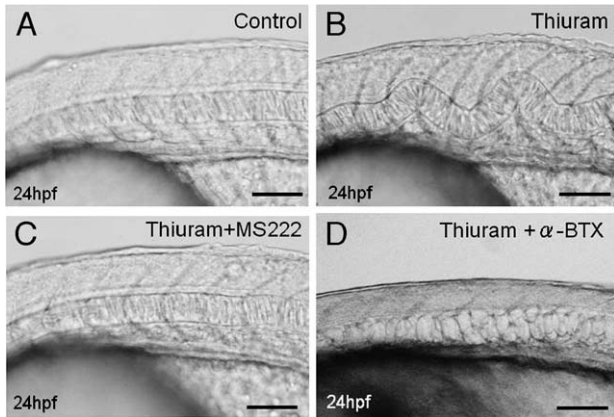


Fig. 5. Pharmacological inhibition of spontaneous contractions masks notochordal response to thiuram. All thiuram-exposed embryos were continuously exposed to 20 nM thiuram beginning at 3 hpf. All images were acquired at 24 hpf and representative embryos are illustrated. (A) Control, non-exposed embryo. (B) Thiuram-exposed embryo displaying characteristic 24 hpf notochord distortion. (C) Thiuram-exposed embryo also exposed to 0.04% tricaine (MS222) from 17 hpf until observation at 24 hpf. (D) Thiuram-exposed embryo also injected with 100 pL of 0.25 mM α -bungarotoxin (α -BTX) at 10–14 hpf. Scale bars: 100 μ m.

are spawned, 25% of the offspring will be homozygotic mutants and paralyzed, 25% will have two normal alleles and 50% should be heterozygotic showing no phenotype since the *sop* mutation is recessive. When clutches of embryos were exposed to 20 nM thiuram, 22% of the 24 hpf embryos had notochords with near-normal morphology (Fig. 6). Video analysis was used to screen the exposed embryos for their spontaneous behavior. The average number of bends per minute was calculated over a 5-min time period to phenotypically sort the embryos. All of the embryos with normal notochords possessed no spontaneous contractions (*sop*^{-/-}). The embryos with severely distorted

notochords on the other hand, had the typical contralateral behavior (*sop*^{+/+} or ^{+/-}). Taken together, these three independent approaches indicate that trunk muscular contractions are absolutely necessary to reveal the developmental deficit induced by dithiocarbamate exposure.

Histology of dithiocarbamate-exposed embryos

To evaluate the impact of thiuram on trunk development, ultrastructure and histological analyses were conducted in 17 and 24 hpf control and thiuram-exposed embryos. The ultrastructure of muscle fibers in thiuram-treated embryos was indistinguishable from controls (Figs. 7C and D). The notochord cells of thiuram-exposed embryos contained irregularly arranged nuclei compared to controls. In addition, the notochord sheath is notably thicker in thiuram-exposed, compared to control embryos (Figs. 7A and B). It is important to note that at 17 hpf, the gross notochord morphology is similar in control and thiuram-exposed embryos, and these subtle effects precede the development of the wavy notochord. Cells undergoing apoptosis or necrosis were not detected in the trunk sections at either the 17- or 24-h developmental time points, irrespective of the presence or the absence of thiuram. This was confirmed by whole-mount TUNEL staining (data not shown).

In thiuram-exposed embryos, distorted notochords possessed irregular and disarranged vacuoles in the trunk of 24 and 30 hpf embryos, confirming the observations made by live microscopic imaging. Vacuoles, present in both control and thiuram-exposed embryos, contained glycogen particles. The glycogen particles were more extensively condensed in notochordal cells of thiuram exposed embryos at 17 hpf prior to vacuole formation compared to the control embryos (Fig. 7B). When measured with an F-kit (Roche

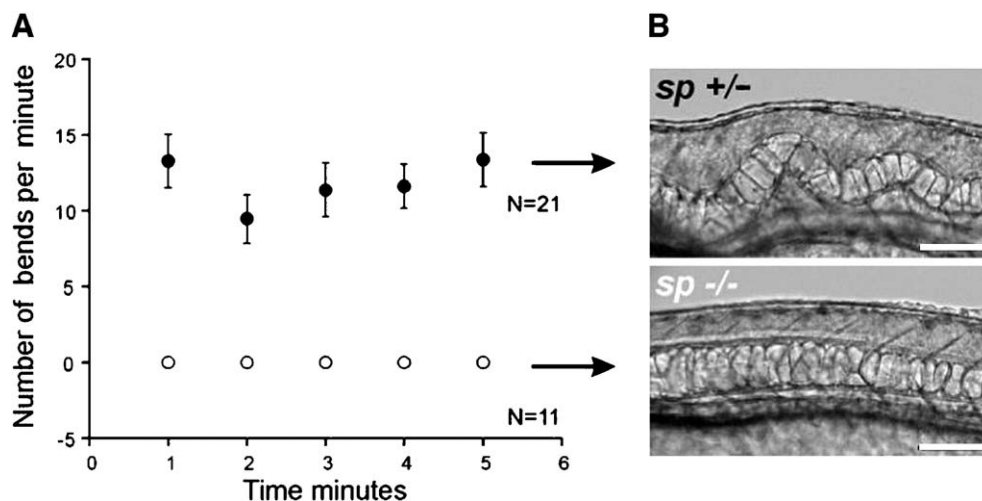


Fig. 6. Paralytic mutants fail to display notochord distortions in response to thiuram. The offspring from heterozygotic *sop* spawning pairs were exposed to 20 nM thiuram beginning at 3 hpf and continued through 24 hpf. (A) The embryonic phenotypes of the clutches were used to deduce genotype. (B) Representative images of embryonic response to thiuram with the indicated genotypes. Scale bars: 50 μ m.

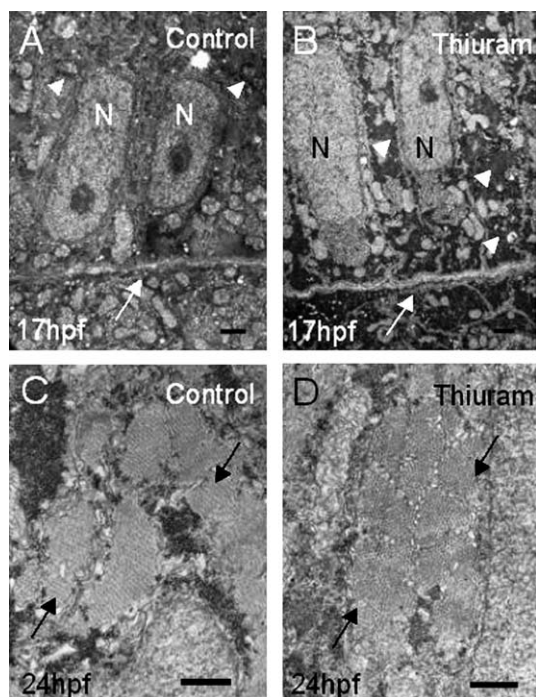


Fig. 7. Subtle effects of thiuram on trunk ultrastructure. (A) Notochords of control and (B) 20 nM thiuram-exposed embryos at 17 hpf, (C) myotomes of control and (D) 20 nM thiuram-exposed embryos at 24 hpf. At 17 hpf, immature notochord cells contain accumulations of glycogen (arrow heads), which are much apparent in thiuram-treated embryo (A). Notochord sheaths (arrows) are slightly thicker in thiuram-treated embryo than control (compare panels A and B). In myotomes of 24 hpf, the densities of myofibril and arrangement of actin and myosin filament (arrows) are similar between thiuram-treated and control embryos (C and D). Scale bars in panels A and B: 1 μm ; scale bars in panels C and D: 0.5 μm .

Diagnostics, Switzerland), glycogen content in thiuram-treated embryos was not significantly different from that in control embryos at 17 hpf, irrespective of the presence or the absence of the yolk, a glycogen reservoir [(with yolk, $n = 3$) control 1118.9 ± 283.7 ng/embryo, thiuram-treated 1165.9 ± 303.9 ng/embryo; (without yolk, $n = 3$) control 72.4 ± 33.8 ng/embryo, thiuram-treated 55.7 ± 21.3 ng/embryo]. This may suggest abnormal carbohydrate transport in the notochord of 20 nM thiuram-treated embryos.

Collagen and laminin in notochord under thiuram exposure

As the above observations suggested that the structure of the trunk could be changed by thiuram, we focused on the extracellular matrix in the trunk. The zebrafish notochord selectively expresses collagen type 2a (col2a) among various types of collagen until 24 hpf (Yan et al., 1995; Fig. 8). Using whole-mount in situ hybridization techniques, it was revealed that the expression of col2a mRNA in thiuram-exposed and unexposed embryos at 17 hpf was indistinguishable (Figs. 8A and C). Although this was nearly the case for 24 hpf embryos, there were notable gaps in col2a mRNA expression in thiuram-exposed embryos (Fig. 8D). *Sleepy*, a previously characterized zebrafish

mutant, has a notochord morphology that is similar to that of dithiocarbamate exposed embryos. *Sleepy* embryos have wavy notochord and disarranged somites (Stemple et al., 1996). Recently, it has been reported that *sleepy* is a defect in laminin, which constitutes the basal membrane and is localized in somites and in the notochord of zebrafish (Parsons et al., 2002). Using immunohistochemistry with anti-laminin antibody, the expression pattern of laminin was not impacted by thiuram (data not shown).

Molecular marker expressions in somites and notochord under thiuram exposure

Using whole-mount in situ hybridization, a number of well-characterized molecular markers were evaluated to determine if thiuram exposure altered notochord or muscle development. A number of markers were used to evaluate myogenesis. For example, the *mesp* bHLH transcription factors are important in establishing presomitic mesoderm patterning (Sawada et al., 2000). There was no marked difference in mesoderm posterior *mesp a* or *mesp b* mRNA expression at 18 hpf in control and thiuram-exposed embryos. *Myo D*, an early muscle marker (Weinberg et al., 1996), was also similarly expressed at 10, 12, and 24 hpf in control and thiuram-treated embryos (data not shown). Although *engrailed-1* was similarly transcribed in muscle pioneer in thiuram-treated embryos (data not shown), sonic hedgehog (*shh*) had a more intense distribution pattern in 24-h thiuram-exposed embryos compared to unexposed embryos. No tail (*ntl*), is a zebrafish homologue of brachyury and is indispensable for notochord formation. It is well established that *ntl* expression is restricted to the notochord and its expression gradually decreases between 14 and 48 hpf (Yamakoshi and Shimoda, 2003). Thiuram exposure results in a more intense notochordal distribution of *ntl* transcripts in 24 hpf embryos (compare Figs. 9B and D).

Effects of metal chelating agents on zebrafish embryos

It is well accepted that dimethyl dithiocarbamates including thiuram, ziram, and disulfiram chelate cationic metals including Fe^{2+} and Cu^{2+} . To begin to test the hypothesis that thiuram may be leading to the developmental effects via a chelation mechanism, we investigated the effects of other chelating agents on zebrafish development. Application of two Cu^{2+} chelators, D-penicillamine (0.1–1 mM) and *N*-phenylthiourea (PTU, 200 μM), beginning at 3 hpf did not affect trunk development (data not shown). It is well established that PTU exposure inhibits tyrosinase activity through Cu^{2+} chelation (Williamson, 1997), and is routinely used by researchers to facilitate embryonic imaging (Westerfield, 1995). PTU effectively blocked pigmentation of embryos, indicating that PTU was absorbed by embryos and exerted Cu^{2+} chelating action. In relation to this, it should be noted that 20 nM thiuram did not affect pigmentation, but 1000 nM thiuram completely abolished

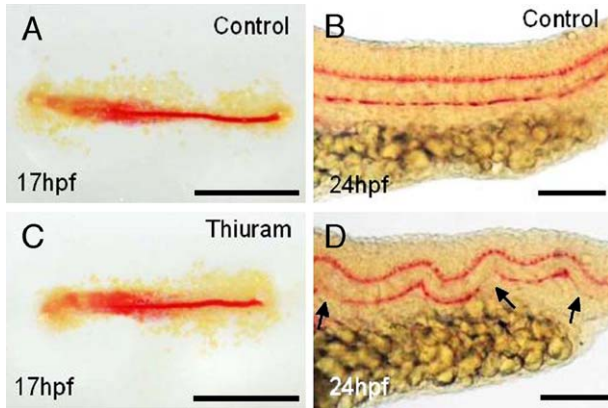


Fig. 8. Collagen type 2a (*col2a*) expression. Control and thiuram-exposed embryos (20 nM) were fixed at 17 hpf (A, C) and 24 hpf (B, D) for whole-mount in situ hybridization with a collagen type 2a specific probe. Regions of *col2a* expression appear red. Arrows in panel D indicate gaps of expression of collagen type 2a. Scale bars in panels A and C: 400 μ m; scale bars in panels B and D: 100 μ m.

pigmentation (data not shown). When the Fe^{2+} selective chelator defferoxamine was used (0.1–1 mM), the notochord had normal morphology (data not shown). When the other Fe^{2+} selective chelator 2,2'-dipyridyl was used (40–50 μ M), pigmentation was effectively blocked and the notochord developed an undulated morphology (Figs. 10D and E). In addition, the myotome boundaries were dramatically impacted. The characteristic chevron appearance was lost and they instead were vertically arranged relative to the anterior posterior axis. Although not normal, these effects were different than those produced following dithiocarbamate exposure (Fig. 10F). Finally, it is important to note that the 2,2'-dipyridyl-induced trunk malformations were not dependent on spontaneous contractions as tricaine exposure did not impact the 2,2'-dipyridyl response (data not shown).

Discussion

In this study, characteristic wavy notochord, which was originally reported in rainbow trout (van Leeuwen et al., 1986), was produced by thiuram and other dithiocarbamates in zebrafish. All embryos showed complete reproducibility upon treatment with these dithiocarbamates at levels of several tens of nanomolars or above. In addition, all dithiocarbamates examined caused disorganization of somites with shallow angle, and shortening of yolk sac extension and body length, as if the body was compressed front to back. Similar observations have been reported for zebrafish embryos treated with disulfiram (Marsh-Armstrong et al., 1995) and sodium metam (Haendel et al., 2004), and for flounder embryos treated with disulfiram (Suzuki et al., 2001). Thus, these malformations in fish embryos can be regarded as a common toxicological property of dithiocarbamate compounds. Similar notochord

abnormalities caused by thiuram also have been observed in *Xenopus* embryos (Ghate, 1983).

Many developmental toxicants studied in zebrafish lead to systemic edema and mortality, and it is noteworthy that neither occurred following dithiocarbamate exposures in range in these studies (0–1000 nM). A 20-nM waterborne thiuram exposure, which leads to a 100% incidence of distorted notochord, did not grossly impact development. Developmental progression did not appear to be impacted. The rate of somitogenesis, the expression of temporal developmental markers, and the initiation of normal spontaneous contractions were not impaired. It has been proposed that dithiocarbamates are toxic as a result of their general inhibition of sulfhydryl containing enzymes involved in the TCA cycle (Miko and Chance, 1975). Our results do not support this general hypothesis as thiuram was present throughout gastrulation, and primary organogenesis, periods of intense energy demands. These data imply that the effect of dithiocarbamates is rather specific.

By careful observation and imaging, we were able to associate the onset of distorted notochord in thiuram-exposed embryos with the initiation of spontaneous contractions. It is important to emphasize that the notochord morphology and myotome organization was indistinguishable in control and thiuram-exposed embryos before 17 hpf. Just after this, distortions appeared, coincident with the beginning of spontaneous trunk contraction. The zebrafish notochord mutant, *gulliver*, with a similar wavy notochord phenotype, has normal body length and yolk sac extension with longer notochord than wild type (Stemple et al., 1996). This is in striking contrast to our observations of thiuram-treated embryos, in which notochord length is unchanged, while there is an apparent reduction in the rate of overall body lengthening beginning at 17 hpf. These results are consistent with the hypothesis that the underlying deficit

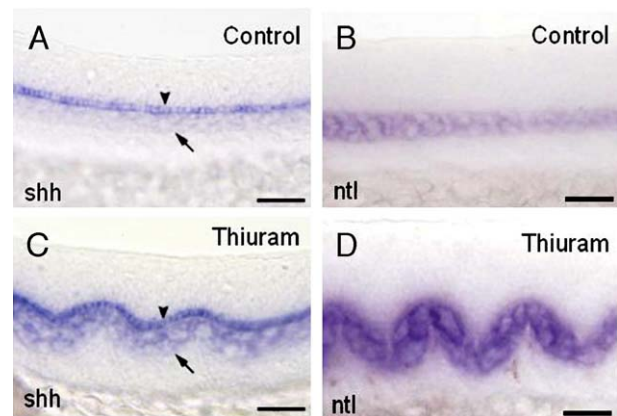


Fig. 9. Expression of notochord markers are impacted by thiuram. Control (A, B) and 20 nM thiuram-treated embryos (C, D) were fixed at 24 hpf for whole-mount in situ hybridization with *shh* (A, C) and *ntl* (B, D). Positive signals from the BM-purple appear blue. Arrow and arrowheads in panels A and C indicate *shh* expressions in the notochord and the floor plate of neural tube, respectively. Scale bars: 100 μ m.

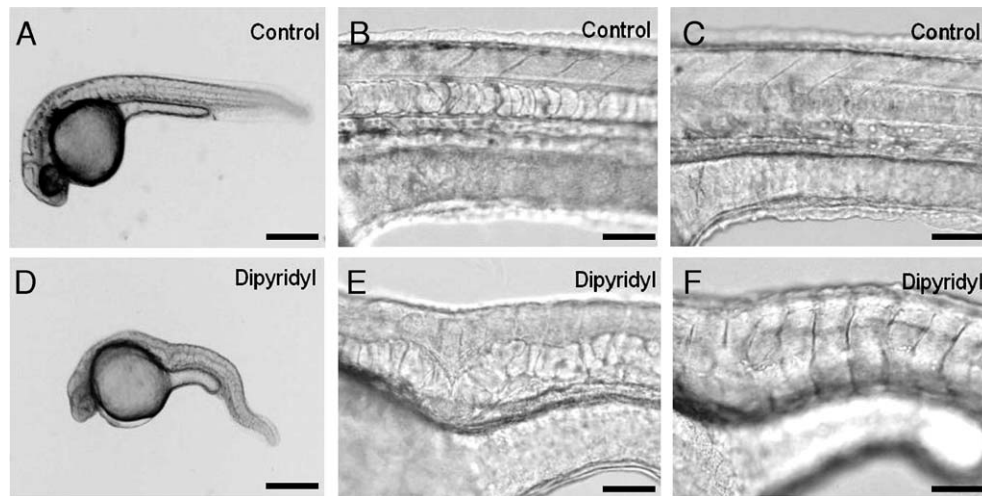


Fig. 10. Embryonic response to divalent metal ion chelation. Morphological disorganizations of zebrafish embryos by Fe^{2+} chelator, 2,2'-dipyridyl. Embryos exposed to 2,2'-dipyridyl (D–F: 45 nM) and non-treated control embryos (A–C) from 10 hpf until observation at 26 hpf. All lateral images of live embryos. (A, D) Whole body; (B, E) focused on notochord in the trunk; (C, F) focused on somites in the trunk. Scale bars in panels A and D: 300 μm ; scale bars in panels B, C, E, and F: 100 μm .

results in the inability of the embryo to maintain proper notochordal and adjacent tissue connectivity. These impaired associations are apparently silent until the developmentally programmed spontaneous contractions expose the deficit. For example, as the notochord necessarily is bent by rhythmic muscle contractions, the notochords of unexposed embryos maintain an intimate association with adjacent tissues, before and after the onset of spontaneous contractions. Therefore, as control embryos grow and lengthen during development, the notochord correspondingly increases in length. In the presence of thiuram, the consequence of impaired associations would not be expected to impact notochordal lengthening; however, in the absence of the rigid notochord scaffold, the trunk would be unable to properly rebound from the contractive forces of the trunk musculature. The most important observation in support of this was that the wavy notochord fails to manifest itself if the spontaneous contractions were inhibited. It is also noteworthy that the other thiuram-dependent endpoints such as myotome morphology, yolk sac extension, and reduced total body length were blocked by tricaine, indicating that these trunk deformations are also contraction dependent. Therefore, it seems that impaired structural interactions between the notochord and surrounding tissues could account for all trunk deformations in thiuram-treated embryos. More detailed studies are necessary to thoroughly test this hypothesis.

The molecular target for dithiocarbamates remains to be identified but it could conceivably reside in the muscle, the notochord, or adjacent connective tissue. The onset of spontaneous trunk contraction and their frequency appeared normal in the presence of thiuram. Furthermore, the ultrastructure of muscle fibers in thiuram-treated embryos was indistinguishable from controls, and the expression of selected genes involved in somitogenesis was not affected

by dithiocarbamate exposure, before or after deformations. Collectively, these data suggest that early muscle development and innervation are not principal dithiocarbamate targets. In the notochord, on the other hand, subtle changes were detected in some gene markers at 17 hpf, before the onset of deformation, suggesting that notochord development may be impacted by thiuram. Furthermore, ultrastructural observations of the notochord revealed that thiuram exposure leads to impacts on glycogen accumulation and altered arrangement of nuclei. The cause and significance of these subtle alterations remain to be further characterized. Glycogen deposition in human embryonic notochords is normal and speculated to be necessary for energy production in this structure with insufficient mitochondria (Murakami et al., 1985). As there was no detectable difference in total embryonic glycogen content, the increased accumulation of glycogen in thiuram-exposed embryos may represent altered glycogen distribution.

Collagen is an important protein necessary for intra- and extracellular structure; thus, collagen synthesis and metabolism is a potential target for dithiocarbamates. Expression of *col2a*, the only collagen type expressed in notochord of zebrafish by 24 hpf (Yan et al., 1995), was unaffected at time points prior to the development of the distorted notochord in thiuram-exposed embryos. Similar results were obtained with following sodium metam exposure in zebrafish (Haendel et al., 2004). However, alterations in *col2a* mRNA expression were detected at 24 hpf following exposure to both thiuram and sodium metam, albeit with notable differences. In thiuram-exposed animals, there were gaps in the *col2a* expression pattern in the notochord sheath, while in sodium metam-exposed animals, *col2A* was dramatically overexpressed as late as at 36 hpf (Haendel et al., 2004). Alterations in collagen 2a expression could indicate profound effects of thiuram on collagen metabo-

lism. Alternatively, thiuram could affect the maturation process of collagen required for mechanical stabilization in the notochord. Both Marsh-Armstrong et al. (1995) and Suzuki et al. (2001) found that disulfiram produced wavy notochord malformation in zebrafish and flounder embryos, when it was used to inhibit retinal aldehyde dehydrogenase. It has been reported that *neckless*, a zebrafish mutant that lacks retinaldehyde dehydrogenase type 2 (rALDH2) activity, does not have abnormal notochord morphology (Begemann et al., 2001). Therefore, the mechanism by which disulfiram produces notochord distortions is independent of rALDH2 activity. Suzuki et al. (2001) observed no obvious changes in expression of *hoxd-4* and *sonic hedgehog*, both of which are transcriptionally regulated by retinoic acid. It was speculated that disulfiram may inhibit ascorbate dehydrogenase, which is needed for mechanical stabilization of collagen through Cu^{2+} chelation (Wimalasena and Dharmasena, 1994).

There is considerable historical data demonstrating that dithiocarbamates effectively chelate metals; in fact, most commercially available dithiocarbamates are sold as metal-containing complexes. Of the four chelators tested, only 2,2'-dipyridyl partially mimicked the notochordal effects. Furthermore, blockade of spontaneous trunk movement by tricaine abolished wavy notochord caused by thiuram, but did not affect trunk malformation caused by 2,2'-dipyridyl, suggesting different mechanisms for 2,2'-dipyridyl and thiuram-induced trunk malformations. *N*-phenylthiourea, a Cu^{2+} chelator, blocked pigmentation but did not affect normal notochord formation. In the pioneering work with rainbow trout embryos (van Leeuwen et al., 1986), a wavy notochord resulted from zineb and maneb exposure. Zineb and maneb are diethyl dithiocarbamates and it was proposed that mechanistically, they produced toxicity by an oxidative stress-dependent mechanism. Sodium metam and methyl isothiocyanate also induced a similar wavy notochord in zebrafish (Haendel et al., 2004). The common responses to sodium metam, MITC, thiuram, and disulfiram is notable, as MITC is not a dithiocarbamate, and would have a limited capacity to chelate divalent ions. Finally, since divalent ions such as Fe^{2+} and Mn^{2+} are essential for some enzymes involved in energy production within mitochondria, the observation that, thiuram (20 nM) did not show growth retardation, would also not support the hypothesis that cation-chelating activity is the main factor in causing trunk malformation by dithiocarbamates.

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