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Chronic exposure to triadimenol at environmentally relevant concentration adversely affects aging biomarkers in *Caenorhabditis elegans* associated with insulin/IGF-1 signaling pathway

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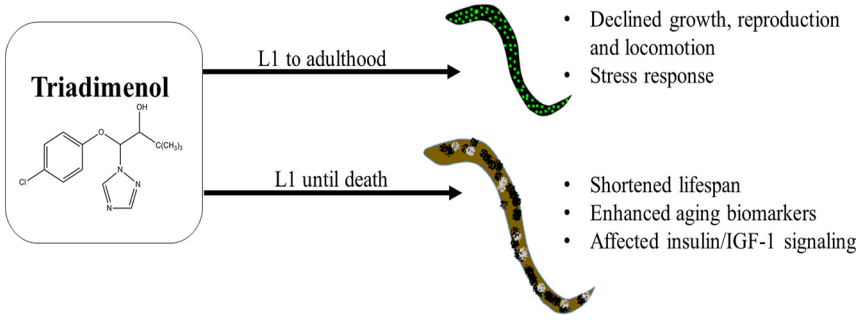
HIGHLIGHTS

- Chronic triadimenol exposure adversely affects growth, reproduction, and locomotive behaviors in *C. elegans*.
- Chronic triadimenol exposure shortens lifespan in *C. elegans*.
- Chronic triadimenol exposure adversely affects age-related behavioral changes, biomarkers in *C. elegans*.
- Chronic triadimenol exposure triggers DAF-16 nuclear localization.
- Chronic triadimenol exposure acts on IIS to affect worms aging.

GRAPHIC ABSTRACT



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ABSTRACT

Triadimenol, an agricultural fungicide, is an emerging environmental concern due to its wide usage, detection in the environment, and its chemical persistency. Triadimenol has been found to disrupt endocrine signaling and alter function of several transcription factors, yet its age-related toxicity effects remain unclear. This study used *Caenorhabditis elegans* as an *in vivo* model organism to elucidate the age-related effects of triadimenol and its underlying mechanisms. The results showed that chronic exposure to triadimenol at environmentally relevant concentrations (3, 30, and 300 μg/L) adversely affected several toxicity endpoints including growth, total brood size, and locomotive behaviors. In addition, triadimenol (300 μg/L) significantly reduced the mean lifespan of wild-type N2 *C. elegans* from 17.9 to 16 days. Chronic exposure to triadimenol (300 μg/L) also significantly affected age-related behavioral changes, with a decreased pharyngeal pumping rate and an increased defecation cycle. Moreover, an increased accumulation of aging biomarkers including lipofuscin, lipid peroxidation, and reactive oxygen species (H_2O_2 and $O_2^{\bullet-}$) level upon chronic triadimenol exposure was observed in aged worms. Furthermore, chronic triadimenol exposure increased the transcriptional factor DAF-16 nuclear localization. Finally, mutation of *daf-2*, *age-1*, *pdk-1*, *akt-1*, or *akt-2* restored the accumulation of lipofuscin in aged worms upon chronic triadimenol exposure, while mutation of *daf-16* led to more enhanced lipofuscin accumulation. Therefore, the insulin/IGF-1 signaling pathway may serve as an important molecular basis for triadimenol induced aging declines in *C. elegans*.

1. Introduction

Azole chemicals are a class of five-membered heterocyclic compounds with a nitrogen atom and at least one other non carbon atom

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in the ring. They are widely used in agriculture and medications due to their excellent fungicidal properties (Chen and Ying 2015). Azoles elicit their fungicidal activity by inhibiting activity of sterol 14-α-demethylase (cytochrome P450 family 51, CYP51) and aromatase (CYP19), leading to the disruption of steroidogenesis, defective membrane integrity, and eventually fungal death (Zarn et al. 2003). Triadimenol, a potent metabolite of triadimefon, is one of the most commonly used fungicides. Triadimenol is mainly used to treat seeds, fruits, and vegetables to prevent mildew and rusts (Leath and Bowen 1989; Liang et al. 2013). The widespread and intensive usage of triadimenol has led to great concern as triadimenol has been detected at low levels in the environment, such as agricultural catchment (3–25 μg/L), rainwater (0.03–1.74 μg/L), and soil (26.1 mg/kg) (Kreuger 1998; Dubus et al. 2000; Pose-Juan et al. 2015). In addition, triadimenol and its metabolites were found in winter wheat at 0.1 mg/kg and 0.33 mg/kg, respectively (EFSA 2016). Triadimenol is persistent in the environment (FAO and WHO, 2011), it may pose chronic low-dose exposure risk to the wildlife, yet the chronic toxicity of triadimenol resulting from low-dose exposure is largely unknown.

Several azole chemicals including triadimenol can bind to human CYP51 *in vitro*, suggesting that azoles may affect non-target organisms (Warrilow et al. 2013). As triadimenol is long implicated as an endocrine disrupting chemical (EDC), most studies have been focusing on its reproductive toxicity, teratogenicity, and developmental toxicity in several non-target organisms such as zebrafish, *Xenopus laevis*, and medaka fish (De la Paz et al. 2017; Groppelli et al. 2005; Chu et al. 2016). In addition, triadimenol could act as dopamine agonist to induce neurobehavioral changes in rats (Walker and Mailman 1996), suggesting a neurotoxicity potential in non-target organism.

Aging is a progressive biological process that features accumulation of age-related biomolecules, including reactive oxygen species (ROS), lipofuscin, and physiological decline as well as elevated probability of death (Collins et al. 2008; Lopez-Otin et al. 2013). Accumulating epidemiology and laboratory animal findings have linked EDCs including pesticides with dysregulated aging process (Gore et al. 2015). EDCs such as bisphenol A has been found to accelerate aging process in the nematode *Caenorhabditis elegans* which is associated with the induction of oxidative stress (Tan et al. 2015). Another type of well-known EDC, di (2-ethylhexyl) phthalate (DEHP), has been reported to shorten the lifespan of *C. elegans* (Pradhan et al. 2018). Although there is increasing evidence on EDCs modification of aging process; nevertheless, the effects of the endocrine disrupting fungicide triadimenol on aging process are not yet understood.

Aging is a carefully regulated and well-conserved process in living organisms and several evolutionarily conserved pathways have been implicated in aging regulation in multiple species (Bitto et al. 2015). The insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS) pathway plays a crucial role in endocrinology of aging (Allard and Duan 2011). In *C. elegans*, the IIS includes DAF-2/IGF-1 receptor, AGE-1/phosphoinositide 3-kinase (PI3K), PDK-1 (3-phosphoinositide-dependent kinase 1), AKT-1, -2/protein kinase B (PKB), and transcription factor DAF-16/Forkhead box O (FoxO) (Murphy and Hu 2013). DAF-16 is the major target of IIS, where DAF-2 activation by insulin or IGF-1 triggers AGE-1-PDK-1-AKT-1/-2 phosphorylation cascade. Consequently, these kinases will phosphorylate and thereby inhibit DAF-16 nuclear localization. The transcription factor DAF-16 regulates a wide array of genes involved in growth, development, metabolism, cell cycle, stress response as well as aging process (Tullet 2015). Thus, environmental chemicals interfering with IIS could be detrimental to living organisms. Environmental toxicants such as arsenite, graphene oxide, and perfluorooctane sulfonate (PFOS) have been reported to adversely affect aging process associated with IIS/DAF-16 in *C. elegans* (Yu et al. 2016; Zhao et al. 2016; Xu et al. 2016). Previous study has implicated interactions between triadimenol and PI3K/AKT signaling in mouse liver after chronic exposure (Nesnow et al. 2009). However, whether triadimenol may interact with IIS during chronic exposure is still unknown.

Herein, this study aims to elucidate the effects of chronic triadimenol exposure at environmental relevant concentrations on aging process in *C. elegans* and its underlying mechanisms. By measuring several well-defined age-related endpoints, including growth, reproduction, locomotive behaviors, behavioral and biochemical changes in aged worms, the effects of chronic triadimenol on aging process in *C. elegans* were determined. Furthermore, the association of chronic triadimenol exposure between the aging biomarker and IIS was deciphered.

2. Material and methods

2.1. Chemicals

Chemicals used in this study were mainly purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA) unless otherwise noted. ROS probes such as C_{11}-bodipy$^{581/591}$, dihydroethidium (DHE), and 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Triadimenol (PESTANAL®, analytical standard) was dissolved in ethanol, and then stored as aliquots at −20 °C in the dark.

2.2. Experimental animal and C. elegans maintaining conditions

The bacteria (*Escherichia coli* OP50) and worm strains were obtained from the *Caenorhabditis* Genetics Center (CGC) (University of Minnesota, MN, USA). *C. elegans* strains used in this study were wild-type N2 (*var* Bristol), CB1370 [*daf-2*(e1370)], TJ1052 [*age-1*(hx546)], GR1307 [*daf-16*(mgDF50)], GR1310 [*akt-1*(mg144)], GR1318 [*pdk-1*(mg142)], VC204 [*akt-2*(ok393)], and TJ356 (zIs356[*daf-16*p::*daf-16*A/B::GFP + *rol-6*(su1006)]). All worms were cultured on nematode growth medium (NGM) with *E. coli* OP50 as a food source under 20 °C, except that CB1370 worms were cultured under 16 °C to prevent dauer formation. Prior to toxicity assays, gravid worms were dissolved using alkaline hypochlorite solution (0.45 M NaOH, 2% HOCl) to obtain synchronized eggs.

2.3. C. elegans growth, reproduction, and locomotive behaviors assays

Wild-type N2 worms were exposed to 0, 3, 30 and 300 μg/L triadimenol from L1 stage under 20 °C in S-basal medium containing *E. coli* OP50 (10^9 cells/mL). Concentration of the solvent ethanol was 0.1% in all exposure groups. For growth analysis, worms were exposed for 3 days to obtain day-0 mature adults and photographed under microscope. The body length of worms was analyzed using imaging software Fiji coupled with Wormsizer macro (Moore et al. 2013; Schindelin et al. 2012). Three replicates were performed for growth assay, and total sample size is >100 worms in each experimental group.

For reproduction assay, worms were harvested 2 days after triadimenol exposure, and then individually picked onto NGM agar with a lawn of *E. coli* OP50. The total offspring was counted daily until egg-laying behavior ceased. Five biological replicates were conducted, and 25 worms were analyzed in each experimental group.

For locomotive behaviors assay, body bends and head thrashes of day-0 adulthood were analyzed. Body bends of the worms were measured for 20 s on sterile NGM agar as described by Koelle and Horvitz (1996). Head thrashes were analyzed for 1 min in K-medium droplet as described by Tsalik and Hobert (2003). At least three biological replicates were performed, and at least 100 worms for body bends and 30 worms for head thrashes were analyzed in each experimental group.

2.4. C. elegans lifespan assay

Wild-type N2 worms were exposed to solvent control or 300 μg/L triadimenol from L1 in S-basal under 20 °C for 3 days. Day-0 adults were washed and cultured on control or triadimenol containing NGM agar, with UV-killed *E. coli* OP50 lawn as a food source. The survival of