NeuroToxicology 32 (2011) 331–341

Contents lists available at ScienceDirect

NeuroToxicology

Exposure to Mn/Zn ethylene-*bis*-dithiocarbamate and glyphosate pesticides leads to neurodegeneration in *Caenorhabditis elegans*

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ARTICLE INFO

Article history:
Received 29 September 2010
Accepted 8 February 2011
Available online 3 March 2011

Keywords:
Glyphosate
Mancozeb
C. elegans
Pesticides
NW1229
Neurodegeneration

ABSTRACT

Epidemiological evidence suggests positive correlations between pesticide usage and the incidence of Parkinson's disease (PD). To further explore this relationship, we used wild type (N2) *Caenorhabditis elegans* (*C. elegans*) to test the following hypothesis: Exposure to a glyphosate-containing herbicide (TD) and/or a manganese/zinc ethylene-*bis*-dithiocarbamate-containing fungicide (MZ) may lead to neurotoxicity. We exposed N2 worms to varying concentrations of TD or MZ for 30 min (acute) or 24 h (chronic). To replicate agricultural usage, a third population was exposed to TD (acute) followed by MZ (acute). For acute TD exposure, the LC_{50} = 8.0% (r^2 = 0.6890), while the chronic LC_{50} = 5.7% (r^2 = 0.9433). Acute MZ exposure led to an LC_{50} = 0.22% (r^2 = 0.5093), and chronic LC_{50} = 0.50% (r^2 = 0.9733). The combined treatment for TD + MZ yielded an LC_{50} = 12.5% (r^2 = 0.6367). Further studies in NW1229 worms, a pan-neuronally green fluorescent protein (GFP) tagged strain, indicated a statistically significant ($p < 0.05$) and dose-dependent reduction in green pixel number in neurons of treated worms following each paradigm. This reduction of pixel number was accompanied by visual neurodegeneration in photomicrographs. For the dual treatment, Bliss analysis suggested synergistic interactions. Taken together, these data suggest neuronal degeneration occurs in *C. elegans* following treatment with environmentally relevant concentrations of TD or MZ.

1. Introduction

1.1. Pesticide usage and Parkinson's disease (PD)

Epidemiological studies support a relationship between pesticide usage and Parkinson's disease (PD) (Ascherio et al., 2006; Hancock et al., 2008; Tanner et al., 1999); however, it is unclear as to which pesticides and what mechanisms of action may contribute to the disease's etiology. Other studies suggest that occupational exposure to various metals, including iron (Fe) or manganese (Mn), is risk a factor for PD and/or parkinsonism (Gorell et al., 2004; Hernandez et al., 2002; Racette et al., 2005). Additionally, it is well-documented that various mitochondrial complex I inhibitors induce parkinsonian symptoms in humans and animals (Keeney et al., 2006; Piccoli et al., 2008; Testa et al., 2005). Taken together, data from these studies suggest that pesticides containing Mn (Ferraz et al., 1988; Israeli et al., 1983a,b) or Fe, or those capable of inhibiting mitochondrial respiration (Astiz et al., 2009; Liou et al., 1997; Richardson et al., 2005), may be putative contributors to the onset of neurodegeneration associated with PD.

1.2. Model organism: Caenorhabditis elegans

One difficulty of studying mechanisms of pesticide toxicity *in vivo* is the complexity of most organisms. Although rodents and non-human primates have been used with much success in toxicology studies, downsides include the significant cost of maintaining them throughout their life span, from 14 months to many years, respectively. As parkinsonism is associated with increasing age, this makes chronic, low-level exposure studies difficult and expensive. Recently the nematode *C. elegans* has gained popularity in neurotoxicology testing (Leung et al., 2008; Peterson et al., 2008). While a handful of labs (Easton et al., 2001; Ruan et al., 2009; Saffih-Hdadi et al., 2005; Saha et al., 2009; Svendsen et al., 2010) have examined the gross toxicity of pesticides in *C. elegans*, to our knowledge no labs have looked in this model system at specific neurodegeneration related to PD following exposure to various herbicides or fungicides.

Such studies are greatly simplified in *C. elegans*, compared to more traditional animal models, because it is a simple organism whose genome, which has significant homology to the human

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0161-813X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.neuro.2011.02.002

genome, and cell lineage are completely mapped. Additionally, its nervous system is quite simple compared to that of humans and mammals as it contains only 302 (hermaphrodite) or 381 (male) neurons (Chalfie and White, 1988). Other useful features of *C. elegans* include the fact that they are transparent, have a short lifespan, and produce hundreds of offspring in each generation (Wood, 1988). With this in mind, we initially investigated whether *C. elegans* would be an appropriate model for studying a glyphosate-containing herbicide, TouchDown (TD), and a manganese (Mn)-containing fungicide, mancozeb (MZ). Thus, we used N2 (wild-type) worms to determine dose response curves, and later used NW1229 (pan-neuronal green fluorescent protein (GFP) tagged) worms to determine whether exposure to our pesticides of interest could induce regionally specific neurodegeneration.

1.3. Glyphosate-containing herbicides

Glyphosate-containing herbicides are some of the most prevalently used agrochemicals in the world (Woodburn, 2000). In fact, many important food crops (wheat, corn, soybeans) have been genetically modified so they are resistant to these herbicides, providing weed control without crop damage (Dewar, 2009; Gardner and Nelson, 2008). Glyphosate (Fig. 1A), a glycine analogue and active ingredient in RoundUp and TouchDown (TD), is relatively non-toxic (oral LD_{50} = 4320–5600 mg/kg) to rats (Committee, 1979; Worthington, 1983). For pesticide formulations, however, glyphosate is combined with additional chemicals, which are typically listed only as "inert ingredients" and whose toxicity may be unknown in combination with the active ingredient.

For example, a recent study examined the effects of RoundUp or glyphosate alone on mitochondrial oxidative phosphorylation (Peixoto, 2005). This *in vitro* data demonstrated that the commercial formulation, but not glyphosate alone, significantly decreased the ADP/oxygen ratio at concentrations as low as 0.5 mM glyphosate (the lowest concentration tested), suggesting that exposure to the pesticide, but not the active ingredient alone, lead to mitochondrial dysfunction. These data confirm previous reports that commercial formulations of glyphosate inhibit mitochondria (Bababunmi et al., 1979; Olorunsogo and Bababunmi, 1980; Olorunsogo et al., 1979). Since those who use these pesticides are exposed to the commercial formulations, and not pure glyphosate, this gap in the literature indicates that further studies are necessary to examine whether treatment with the pesticide formulation may lead to neurodegeneration similar to that observed in PD.

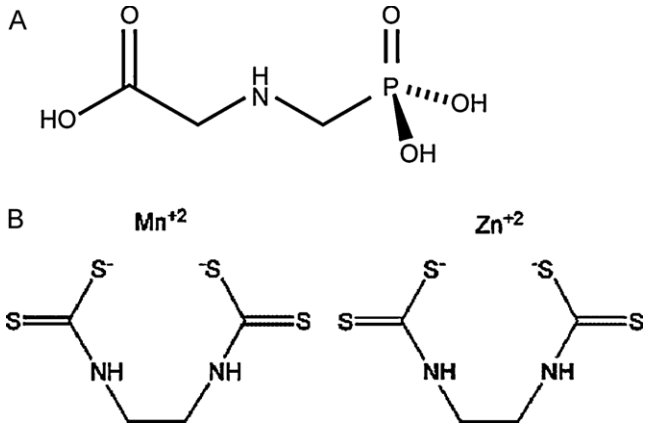

Fig. 1. Chemical structures of pesticide active ingredients. (A) Structure of glyphosate, the active ingredient in the herbicides TouchDown (TD) and RoundUp. (B) Structure of manganese/zinc-ethylene-*bis*-dithiocarbamate (Mn/Zn-EBDC), the active ingredient in mancozeb and mancozate.

1.4. Mn-containing fungicides

Mancozeb (MZ), whose active ingredient is Mn/zinc (Zn)-ethylene-*bis*-dithiocarbamate (Mn/Zn-EBDC; Fig. 1B) is a contact fungicide that inhibits enzyme activity by forming a complex with enzymes involved in ATP production (Cornell, 1987). It is a widely used fungicide, with a total application over the past ten years hovering around 3.6 million kg annually (Gianessi and Reigner, 2006; Giannesse and Marcelli, 2000). Studies indicate that MZ may have pro-apoptotic effects (Calviello et al., 2006), induce neurodegeneration in the nigrostriatal dopamine system, and lead to subsequent vulnerability to additional environmental toxicants (Domico et al., 2007, 2006; Soleo et al., 1996). Data also suggest that, like glyphosate-containing herbicides, MZ inhibits mitochondrial respiration (Domico et al., 2006; Zhang et al., 2003).

1.5. Overview of treatment paradigms

In order to investigate the hypothesis that the commercial formulation of these pesticides (TD and MZ), and not simply their active ingredient(s), may contribute to neurodegeneration, we initially established LC_{50}s for acute and chronic treatments of N2 *C. elegans* with TD or MZ. As most agricultural applications include exposure to multiple pesticides, we also examined *C. elegans* acutely treated with TD followed by an acute exposure to MZ. This latter paradigm is based on the order in which farmers or other agricultural workers are exposed to these chemicals during growing seasons. Lastly, we repeated the exposures in NW1229 worms to determine whether TD and/or MZ contribute to general neurodegeneration in this model organism.

As practically no data relating pesticide usage to neurodegeneration have been generated in *C. elegans*, the focus of the current work was to determine whether TD and/or MZ could produce toxicity in *C. elegans* at environmentally relevant concentrations, and to determine whether neurodegeneration was apparent at the same concentrations. Thus, this work provides systematically established concentration parameters for the commercial formulations of TD and MZ, as well as sets the stage for delineation of potential mechanisms of general toxicity or neurotoxicity in the model organism *C. elegans*.

2. Materials and methods

2.1. Worm and Escherichia coli strains

Wild-type *C. elegans* (N2) and NW1229 worms, as well as NA22 *E. coli* and OP50 *E. coli* (an uracil auxotroph) were provided by the *Caenorhabditis* Genetics Center (CGC), which is funded by the National Institutes of Health (NIH) National Center for Research Resources (NCRR). NW1229 expresses a pan-neuronal green fluorescent (GFP) pattern due to the integration of Ex[F25B3.3::GFP; dpy-20(+)] in dpy-20(−) background.

2.2. Worm maintenance and treatment

Worms were maintained at 20 °C and synchronized according to standard protocols (Brenner, 1974). Briefly, gravid worms grown on 8P plates (51.3 mM NaCl, 25.0 g bactoagar/L, 20.0 g bactopeptone/L, 1 mM $CaCl_2$, 0.5 mM potassium phosphate buffer (pH 6), 0.013 mM cholesterol (in 95% ethanol), 1 mM $MgSO_4$) with a lawn of NA22 *E. coli* (grown in 16 g tryptone/L, 10 g yeast extract/L, 85.5 mM NaCl), were synchronized by exposure to a solution of sodium hypochlorite (4.0 mM) and sodium hydroxide (0.5 mM), and monitored under the microscope until worms released eggs. Approximately 18 h following isolation and purification of eggs, synchronous L2 *C. elegans* were exposed to varying concentrations