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Research Paper

# Chronic exposure to a glyphosate-containing pesticide leads to mitochondrial dysfunction and increased reactive oxygen species production in *Caenorhabditis elegans*

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ABSTRACT

Glyphosate-containing herbicides are among the most widely-used in the world. Although glyphosate itself is relatively non-toxic, growing evidence suggests that commercial herbicide formulations may lead to increased oxidative stress and mitochondrial inhibition. In order to assess these mechanisms *in vivo*, we chronically (24 h) exposed *Caenorhabditis elegans* to various concentrations of the glyphosate-containing herbicide TouchDown (TD). Following TD exposure, we evaluated the function of specific mitochondrial electron transport chain complexes. Initial oxygen consumption studies demonstrated inhibition in mid- and high-TD concentration treatment groups compared to controls. Results from tetramethylrhodamine ethyl ester and ATP assays indicated reductions in the proton gradient and ATP levels, respectively. Additional studies were designed to determine whether TD exposure resulted in increased reactive oxygen species (ROS) production. Data from hydrogen peroxide, but not superoxide or hydroxyl radical, assays showed statistically significant increases in this specific ROS. Taken together, these data indicate that exposure of *Caenorhabditis elegans* to TD leads to mitochondrial inhibition and hydrogen peroxide production.

## 1. Introduction

The increasing adoption and planting of genetically modified, herbicide-resistant agricultural crops (*e.g.*, corn, soy, and wheat) has been mirrored by an increase in the overall amount of glyphosate-containing herbicides applied in these occupational settings (Benbrook, 2012). While pesticides are used by many populations (landscape professionals, home owners, small-scale family gardeners), they are primarily applied by workers in the world's agricultural industry (Grube et al., 2011). Furthermore, the leading class of pesticides consists of herbicide formulations that contain glyphosate (Grube et al., 2011).

Previous studies focused predominantly on the toxicity of the active ingredient, glyphosate, which is relatively non-toxic in both rats (oral $LD_{50}$ = 2 g/kg) and mice (oral $LD_{50}$ = 10 g/kg) (Tomlin, 2006). In light of this low toxicity, research has shifted from assessing glyphosate toxicity alone to determining the toxicity of formulations to which agricultural and industrial workers are actually exposed (de Liz Oliveira Cavalli et al., 2013; Mesnage et al., 2014; Peixoto, 2005). Studies with the agricultural formulation have demonstrated that it is much more toxic than either glyphosate alone or the putative relevant surfactants (Kim et al., 2013). This research, however, was typically performed *in vitro* or in isolated organelles rather than *in vivo*.

It is well-documented that occupational pesticide exposure is associated with an increased risk for some chronic neurodegenerative diseases (*i.e.*, Parkinson's disease (Allen and Levy, 2013); Alzheimer's disease (Baltazar et al., 2014)). In light of reports of greater potency resulting from the readily available products, we sought to investigate whether mitochondrial inhibition or oxidative stress resulted from TD exposure, and could potentially explain the neurodegeneration we previously reported in *C. elegans* treated with TD (Negga et al., 2011;

*Abbreviations:* TD, TouchDown; ROS, reactive oxygen species; *C. elegans*, *Caenorhabditis elegans*; *E. coli*, *Escherichia coli*; CGC, caenorhabditis genetics center; GFP, green fluorescent protein; GST-4p, glutathione-S-transferase 4 promoter; NGM, worm growth media; TMRE, tetramethylrhodamine ethyl ester; DMSO, dimethyl sulfoxide; DHE, dihydroethidium; HPF, hydroxyphenyl fluorescein; ANOVA, analysis of variance

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Negga et al., 2012). In those studies, worms treated chronically with concentrations of TD also used in this research showed neurodegeneration in both dopaminergic (DAergic) and GABAergic neurons. Degeneration of these neuron populations are involved in numerous chronic diseases (Coune et al., 2013; Kalia et al., 2013; Rousseaux et al., 2012; Schwab et al., 2013; Wang et al., 2010). Since the neurodegeneration is often attributable to mitochondrial inhibition and/or oxidative stress (Bhat et al., 2015; Kamat et al., 2014; Mostafalou and Abdollahi, 2013; Okazawa et al., 2014; Piccoli et al., 2008), we wanted to determine if TD exposure would result in modulation of these endpoints.

## 2. Materials and methods

### 2.1. Worm and Escherichia coli strains

Wild-type (N2) and CL2166 worms, as well as NA22 *Escherichia coli (E. coli)* and OP50-1 *E. coli* were obtained from the *Caenorhabditis* Genetics Center (CGC). In CL2166 worms (dvIs19 [(pAF15)*gst-4p*:*gfp*:NLS] III), an oxidative stress-inducible green fluorescent protein gene (*gfp*) is fused with the glutathione-*S*-transferase 4 promoter (*gst-4p*) region (http://www.wormbase.org/db/get?name=cl2166;class=strain).

### 2.2. Synchronization

Protocols for synchronization, treatment, and endpoint assays used in our lab were previously published in detail (Bailey et al., 2016; Todt et al., 2016). Briefly, *C. elegans* were grown at 20 °C on 8P plates (51.3 mM NaCl, 25.0 g bactoagar/L, 20.0 g bactopeptone/L, 1 mM $CaCl_2$, 500 μM $KH_2PO_4$ (pH 6), 13 μM cholesterol (95% ethanol), and 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) until gravid. Synchronization was accomplished by isolating eggs via hypochlorite treatment. Once eggs were removed, they were washed and incubated at 20 °C in an M9 buffer (20 mM $KH_2PO_4$, 40 mM $Na_2HPO_4$, 68 mM NaCl) for 18 h.

### 2.3. Treatments

Worms were exposed to TD following established protocols (Negga et al., 2011) that have been extensively described (Bailey et al., 2016). Briefly, 5000 worms/treatment group n = 3 treatment groups/TD concentration/synchronization were exposed to 2.7%, 5.5%, or 9.8% glyphosate as TD (TouchDown® Hitech, formulation of 52.3% glyphosate, Syngenta AG, Wilmington, DE). These concentrations were used previously (Negga et al., 2011; Negga et al., 2012), and are within application limits recommended by herbicide manufacturers. In order to enable comparison of these results with similar herbicide studies, herbicide concentrations were normalized to percent active ingredient in the sample (glyphosate) rather than total percent of the parent pesticide formulation.

Following exposure to TD for 30 min, washed worms were poured onto nematode growth medium plates (NGM plates; 51.3 mM NaCl, 17.0 g bactoagar/L, 2.5 g bactopeptone/L, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 500 μM $KH_2PO_4$ (pH 6.0), 12.9 mM cholesterol (95% ethanol), 1.25 mL nystatin/L, 200 mg streptomycin/L) with a lawn of OP50-1 *E. coli* for an additional 24 h at 20 °C. Since concentrated TD is diluted with $H_2O$ in agricultural settings, $H_2O$ was used as the control treatment for each study reported here. Following the treatments, worms were assessed for either mitochondrial function or reactive oxygen species (ROS) production.

### 2.4. Mitochondrial assays

#### 2.4.1. Polarographic measurements

All endpoint assays have been previously described in detail (Todt et al., 2016). Briefly, all treatment groups were standardized to yield 10,000 live worms/mL/treatment concentration. Using an oxygen probe (YSI 5304), oxygen measurements were recorded every ten seconds for five minutes at 22 °C (water bath chambers YSI 5301 B Standard Bath).

#### 2.4.2. Proton gradient integrity

For these studies, worm solutions were standardized to 1000 worms/mL/treatment group. Worms from each treatment group were incubated with a final concentration of 50 μM tetramethylrhodamine ethyl ester (TMRE; Biotium, Hayward, CA) in dimethyl sulfoxide (DMSO; final concentration of 0.5% DMSO) for 1 h. Following extensive washing, images were taken with a digital camera attached to a fluorescence microscope.

#### 2.4.3. Relative ATP amount

For these studies, worms were counted such that 250 live worms/treatment group were added to each well of a 96-well plate. ATP concentration was determined with the Promega Mitochondrial ToxGlo™ Assay (Promega Corporation, Madison, WI). Sodium azide was used as a negative control. Fluorogenic peptide substrate (obtained from the assay kit) was added to each well, and incubated at 20 °C for 60 min to determine viability. Fluorescence was measured per the guidelines provided in the assay kit. Afterwards, worms were incubated for 15 min with the luciferin-based ATP probe. Luminescence was measured as previously reported (Todt et al., 2016).

### 2.5. Reactive oxygen species detection

#### 2.5.1. Superoxide detection

Normalized worm solutions were prepared to yield 5000 live worms/mL/treatment group. Worm were then incubated for three hours in 250 μM dihydroethidium (DHE; Merck KGaA, Darmstadt, Germany). During the incubation period, pictures were taken by placing three 50 μL samples per treatment group on a UV light box (UVP Benchtop 2UV Transilluminator, Upland, CA) at a wavelength of 302 nm.

#### 2.5.2. Hydrogen peroxide detection

For these studies, a total of 200 worms/treatment group/well (in a 96-well plate) were treated with 50 μL of the Amplex® Red (Life Technologies, Grand Island, NY) as previously described (Todt et al., 2016). Negative and positive controls were generated per the protocol accompanying the reagent kit. Aluminum foil-covered plates were incubated for 1 h, at which time fluorescence was assessed as indicated in the kit instructions.

#### 2.5.3. Hydroxyl radical detection

Treated worms were normalized to 1000 live worms/mL/treatment group. The hydroxyphenyl fluorescein (HPF) probe (Life Sciences, Grand Island, NY) was added, and the solutions incubated for 1.5 h at 20 °C per published protocols (Todt et al., 2016). Fluorescence associated with 250 live worms was then determined using a Promega® GloMax-Multi + Detection System.

#### 2.5.4. Glutathione-S-transferase (GST) up-regulation

Glutathione-*S*-transferase (GST) facilitates the conjugation of reduced glutathione to electrophilic xenobiotics. In *C. elegans*, up-regulation of GST has been used to assess oxidative stress. In order to determine if GST transcription and translation occurred post-TD treatment, CL2166 worms (dvIs19 [(pAF15)*gst-4p*:*gfp*:NLS] III) were photographed using a digital camera attached to a fluorescence microscope (see *Fluorescence microscopy* section).