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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.

Keywords

Glyphosate; *C. elegans*; mitochondrial inhibition; reactive oxygen species; hydrogen peroxide; herbicide

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1.0 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 LD₅₀ = 2 g/kg) and mice (oral LD₅₀ = 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; 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.*, 2016; 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.

2.0 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 bactopectone/L, 1 mM CaCl₂, 500 μM KH₂PO₄ (pH 6), 13 μM cholesterol (95% ethanol), and 1 mM MgSO₄) 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 KH₂PO₄, 40 mM Na₂HPO₄, 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, 5,000 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 bactopectone/L, 1 mM CaCl₂, 1 mM MgSO₄, 500 μM KH₂PO₄ (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 H₂O in agricultural settings, H₂O 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 5301B Standard Bath).

2.4.2 Proton gradient integrity—For these studies, worm solutions were standardized to 1,000 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 5,000 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 1,000 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).

2.6 Fluorescence microscopy

Photomicrographs were taken per published protocols (Negga *et al.*, 2012; Todt *et al.*, 2016). A Leitz & Wetzlar (Halco Instruments, Inc) microscope equipped with a 50-W AC mercury source lamp (E. Leitz, Rockleigh, NJ) and 40× objective (Leitz & Wetzlar, Halco Instruments, Inc.) was coupled to a digital camera (Micrometrics, MilesCo Scientific, Princeton, MN) operated by Micrometrics software (SE Premium, v2.7). Photomicrographs were analyzed to determine red (TMRE) or green (GST::GFP) pixel intensity.

2.7 Statistical analysis

All data were analyzed using GraphPad Prism (version 6.0 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Polarographic data are presented as mean \pm standard error of the mean (SEM) and represent N = 4 separate synchronizations, with at least 15,000 worms per intra-experimental replication (n = 3). Differences in slope were determined using linear regression followed by one-way analysis of variance (ANOVA) of the regression lines. If the ANOVA resulted in an overall significant p value (p < 0.05), a *post-hoc* Tukey's test was performed.

Data the ATP assays are shown as mean \pm SEM and represent N = 3 separate synchronizations with at least 500 worms per intra-experimental replication (n = 3). A one-way ANOVA, followed by a *post-hoc* Dunnett's test, was used to determine statistically significant differences among groups. Red or green pixel intensity of individual worms (n > 5 for each independent replication) was measured TMRE and GST regulation assays. Data are represented as mean \pm SEM and represent N = 3 separate synchronizations, of at least three independent replicates (n = 3). One-way ANOVA, followed by a *post-hoc* Dunnett's test, was used to assess whether statistically significant differences among groups could be attributed to treatment.

3.0 Results

3.1 Overall mitochondrial inhibition

To determine whether glyphosate inhibited mitochondrial function following exposure to TD, oxygen consumption from whole worms was measured over the course of a five-minute assessment period. Oxygen consumption during this period indicated that chronic treatment with 5.5% or 9.8% glyphosate (as TD) resulted in statistically significant decreases in oxygen consumed per time (slope) compared to control (****p < 0.001 and ***p < 0.001, respectively) Figure 1.

3.2 Decreased proton gradient integrity

To further ascertain whether decreased oxygen consumption observed in Figure 1 impaired the ability of the mitochondria to establish a proton gradient, worms were incubated with TMRE after TD treatment. TMRE accumulates within active mitochondria if there is an intact proton gradient. Analysis of red pixels (TMRE fluorescence) was completed, the data indicated 29% fewer red pixels associated with worms in the 9.8% TD group. This difference was statistically significant when compared to control worms (***p < 0.0001) Figure 2.

3.3 Decreased relative ATP levels

Relative ATP concentrations were also measured to see if mitochondrial inhibition resulted in less ATP. Following incubation with the luminescent probe (ATP detection), analysis indicated that TD-exposed worms had around 17% less ATP than control worms (Figure 3A). This, however, did not reach the level of statistical significance. Since lower relative concentrations of ATP could either result from an actual decrease in ATP levels, or a decrease in the number of live worms, we followed up the ATP assessment by determining

activity of a necrotic protease. Results from this assay (Figure 3B) revealed a statistically significant decrease of 27% in relative fluorescence for worms in the 9.8% TD group compared controls (*p < 0.05). This further analysis suggested that decreased ATP in the highest treatment group was likely due to decreased worm viability.

3.4 Superoxide and hydrogen peroxide detection following treatment

Since we observed mitochondrial inhibition, we next sought to identify the particular ROS produced *in vivo*. DHE is oxidized in the presence of $\bullet\text{O}_2$, resulting in the formation of a red fluorescent product. Analysis of red pixel number indicated that there was no statistically significant difference between treatment groups compared to control (Figure 4A).

Since no significant $\bullet\text{O}_2$ production was detected, we next assessed the presence of hydrogen peroxide (H_2O_2). We chose to look at this ROS next because a one-electron transfer to $\bullet\text{O}_2$ could yield H_2O_2 , perhaps explaining the lack of $\bullet\text{O}_2$ in the previous assay. Following exposure of worms to TD, Amplex[®]Red was added to assay tubes as described. Analysis indicated (Figure 4B) that chronic treatment with 5.5% or 9.8% TD resulted in a statistically significant decrease in H_2O_2 production compared to control worms (***p < 0.001).

3.5 Hydroxyl radical detection following treatment

In light of the high H_2O_2 amounts compared to controls, we wanted to determine if hydroxyl radicals ($\bullet\text{OH}$) were also present in detectable levels. This was important because $\bullet\text{OH}$ can be produced from a one-electron transfer to H_2O_2 . To this end, *C. elegans* were incubated with HPF, which oxidizes and fluoresces in the presence of $\bullet\text{OH}$. Analysis indicated there were no statistically significant differences among any of the groups (data not shown).

3.6 GST up-regulation

In light of decreased oxidative stress, as inferred by H_2O_2 assessment (Figure 5), we used a transgenic worm strain, CL2166 (*gst-4p::gfp*), which has a *gfp* construct attached to the promoter region of *glutathione-S-transferase*. When these worms experience increased oxidative stress, *gst-4* transcription and translation is increased, and the worms demonstrate increased fluorescence. Following treatment with TD, green pixel analysis indicated that worms exposed to the two highest concentrations of the herbicide resulted in a statistically significant increase in the number of green pixels compared to controls (***p < 0.001) (Figure 5).

4.0 Discussion

Given that both mitochondrial dysfunction and reactive oxygen species (ROS) production are associated with numerous diseases (Chakraborty *et al.*, 2013; Wang *et al.*, 2013), we wanted to determine whether exposure to TD would also inhibit mitochondrial respiration. We also wanted to investigate the possibility that TD may initiate ROS production. Although mitochondrial respiration studies are often conducted in isolated mitochondrial fractions, we completed these *in vivo* using *C. elegans* to observe the effect TD had on intact mitochondria in a live organism. As *C. elegans* are transparent, we used fluorometric

markers to facilitate visualization of mitochondrial inhibition and ROS levels in whole organisms. While it is the case that this worm is a much simpler model organism than even zebrafish (*Danio rerio*), these worms have many similarities to mammals (Avila *et al.*, 2012; Calahorro and Ruiz-Rubio, 2011). For instance, worms have numerous gene homologs and orthologs associated with various human diseases (Mukherjee *et al.*, 2017; Sutphin *et al.*, 2017; Wang *et al.*, 2017). Critically important to our studies, there is also a high degree of similarity of neurotransmitters, pre-synaptic neurotransmitter transporters, post-synaptic receptors, and catabolic and anabolic enzymes associated with the neurotransmitter neuronal populations in humans (Bargmann and Kaplan, 1998; Chase and Koelle, 2007; Jin, 2005). As such, worms are now widely-used in various toxicology and neurodegenerative studies (Wolozin *et al.*, 2011). More importantly for our current work, they are also sensitive to TD neurotoxicity (Negga *et al.*, 2011; Negga *et al.*, 2012) at concentrations used in occupational, agricultural, and industrial settings (Syngenta, 2010).

Previous research regarding glyphosate alone or a commercially-available herbicide formulation showed that the formulation was significantly more toxic than glyphosate in various cell lines (Benachour and Seralini, 2009; Kim *et al.*, 2013; Mesnage *et al.*, 2014). Furthermore, the increased toxicity was still observed when isolated mitochondria were treated with either glyphosate or the herbicide (Olorunsogo *et al.*, 1979; Peixoto, 2005). Based on these reports and our previous research showing neurodegeneration in *C. elegans* following treatment with a glyphosate formulation (Negga *et al.*, 2011; Negga *et al.*, 2012), we sought to test the hypothesis that mitochondrial inhibition and/or oxidative stress could be possible mechanisms of cell death following exposure to TouchDown (TD; Syngenta). The concentrations used in these studies were consistent with those used in our previous work (Negga *et al.*, 2011), and are also within the range of those which occupational agricultural and pesticide workers would be routinely using. For example, glyphosate formulations sold in ready-to-use formulations are often around 2.7% glyphosate (Monsanto, 2014), which is the lowest concentration we used on *C. elegans*. On the other hand, concentrated formulations are manufactured with 37.0–53.2% glyphosate, with recommended application concentrations ranging from 0.4–2.2% for general field spraying, or from 20–30% for spot spraying (Syngenta, 2010). These latter concentrations are well-above those that induced various levels of neurodegeneration in our previous studies (Negga *et al.*, 2011). Since the focus of our current work was to determine potential mechanisms responsible for the neurodegeneration, we used doses that were (1) within the levels used in agricultural settings, and (2) provided various degrees of neuronal damage in *C. elegans*.

The oxygen consumption studies (Figure 1) provided initial data that indicated worms treated with the two highest concentrations of TD did not respire at the same rate as control worms. While this did not provide specific information for proteins in the electron transport chain, it did suggest that TD could potentially inhibit mitochondria. Thus, our next step was to try to determine whether the observed inhibition also resulted in a compromised proton gradient or ATP production. Results from those studies indicated that decreased oxygen consumption was associated with both. TMRE data (Figure 2) confirmed a decrease in TMRE accumulation at the highest TD concentration, which could have caused the trend for decreased ATP production (Figure 3). On the other hand, we cannot completely rule out the possibility that the decreased ATP levels may be due to increased worm death (Figure 3B).

It is often the case that mitochondrial inhibition is accompanied by increased ROS production. For this reason, we also wanted to determine if we could detect changes in various ROS molecules. Initially we assayed for $\bullet\text{O}_2$ production, since it is one of the major ROS associated with mitochondrial inhibition (Turrens, 1997). Instead, we saw changes in H_2O_2 production (Figure 4B) that strongly correlated with up-regulation of GST-4 (Figure 5). Although *gst-4* encodes putative glutathione-requiring prostaglandin D synthase (Sternberg *et al.*, 2017), its regulation is tightly controlled by SKN-1 and its transcription and translation has been used as an indicator of oxidative stress in *C. elegans* (Detienne *et al.*, 2016; Leiers *et al.*, 2003; Zhang *et al.*, 2014)

Several pesticides, as well as classic toxicants (*e.g.*, 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)(Schober, 2004) used to model Parkinson's disease, are known to inhibit mitochondrial function (Keeney *et al.*, 2006; Testa *et al.*, 2005) and/or increase oxidative stress (McCormack *et al.*, 2005). Additionally, both of these endpoints are often observed in patients with Parkinson's disease (Celardo *et al.*, 2014; Hauser and Hastings, 2013) and other pathologies (Barnham *et al.*, 2004; Lin and Beal, 2006). While genetic mutations are also linked to neurodegeneration (Bagyinszky *et al.*, 2014; Zuo and Motherwell, 2013), there is considerable evidence that exposure to environmental toxicants also correlates with pathogenesis (Bouchard *et al.*, 2010; Dick *et al.*, 2007; Reitz *et al.*, 2011). Due to the wide-spread agricultural and occupational use of glyphosate-containing herbicides, chronic exposure to a mitochondrial inhibitor could render a person more susceptible to neurodegeneration, although parkinsonism following a single exposure to a glyphosate-based herbicide has been reported (Barbosa and Leite, 2001).

Although worms are evolutionarily distant from humans, they provide valuable information that can inform future studies in higher organisms (Teschendorf and Link, 2009). Our current data suggest that chronic exposure to TD likely leads mitochondrial inhibition and increased ROS production. Furthermore, these results occur following exposure to the environmentally relevant formulation actually used in agricultural and occupational settings using concentrations well within those suggested by the manufacturer. Thus, we suggest that additional work is needed in higher model organisms using the pesticide formulations to better characterize potential risks of neurodegeneration.

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Abbreviations

ROS	Reactive oxygen species
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>

<i>E. coli</i>	<i>Escherichia coli</i>
CGC	<i>Caenorhabditis</i> 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
TD	TouchDown

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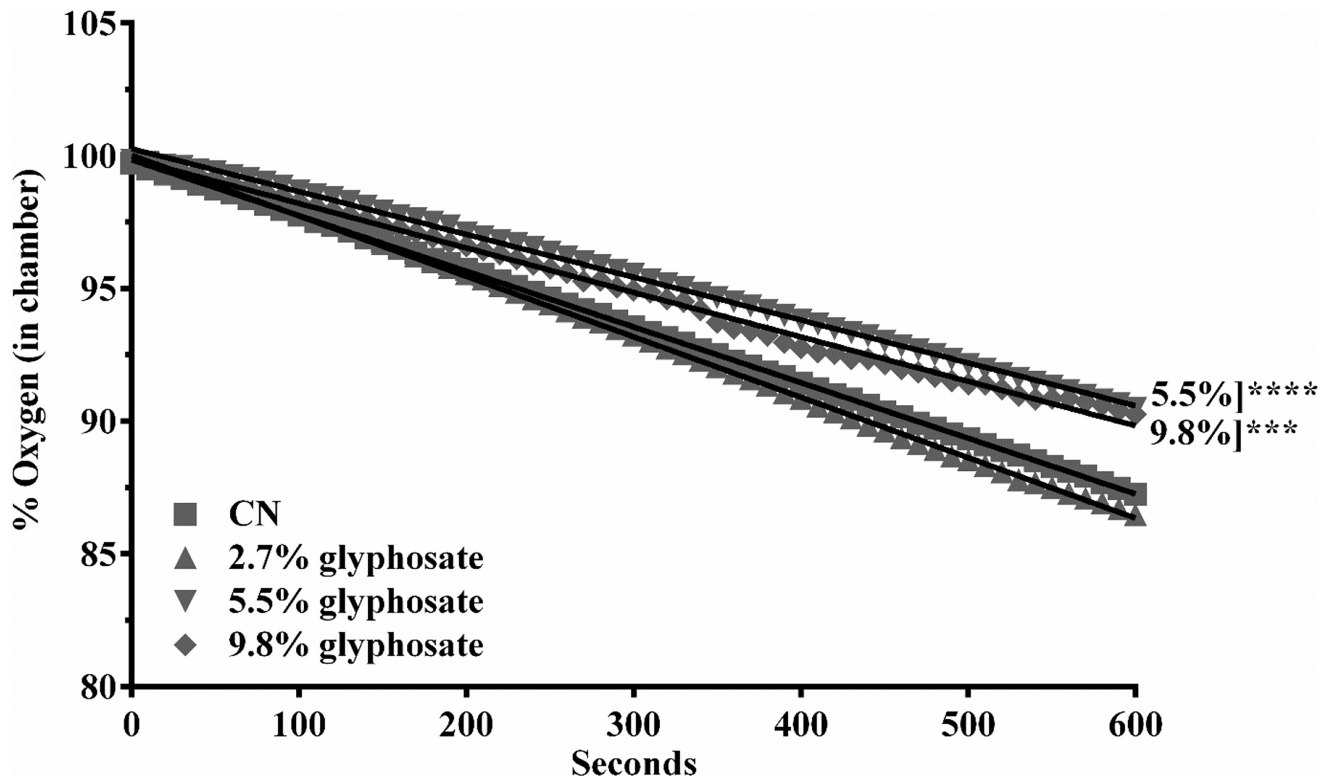


Figure 1.

Decreased mitochondrial respiration following treatment with TD. Following chronic treatment with TD, worms in the mid- and high-concentration groups consistently showed a statistically significant decrease in oxygen consumption. Data are presented as mean percent oxygen consumed and represent N = 4 separate synchronizations. *** $p < 0.001$, or **** $p < 0.001$ compared to controls.

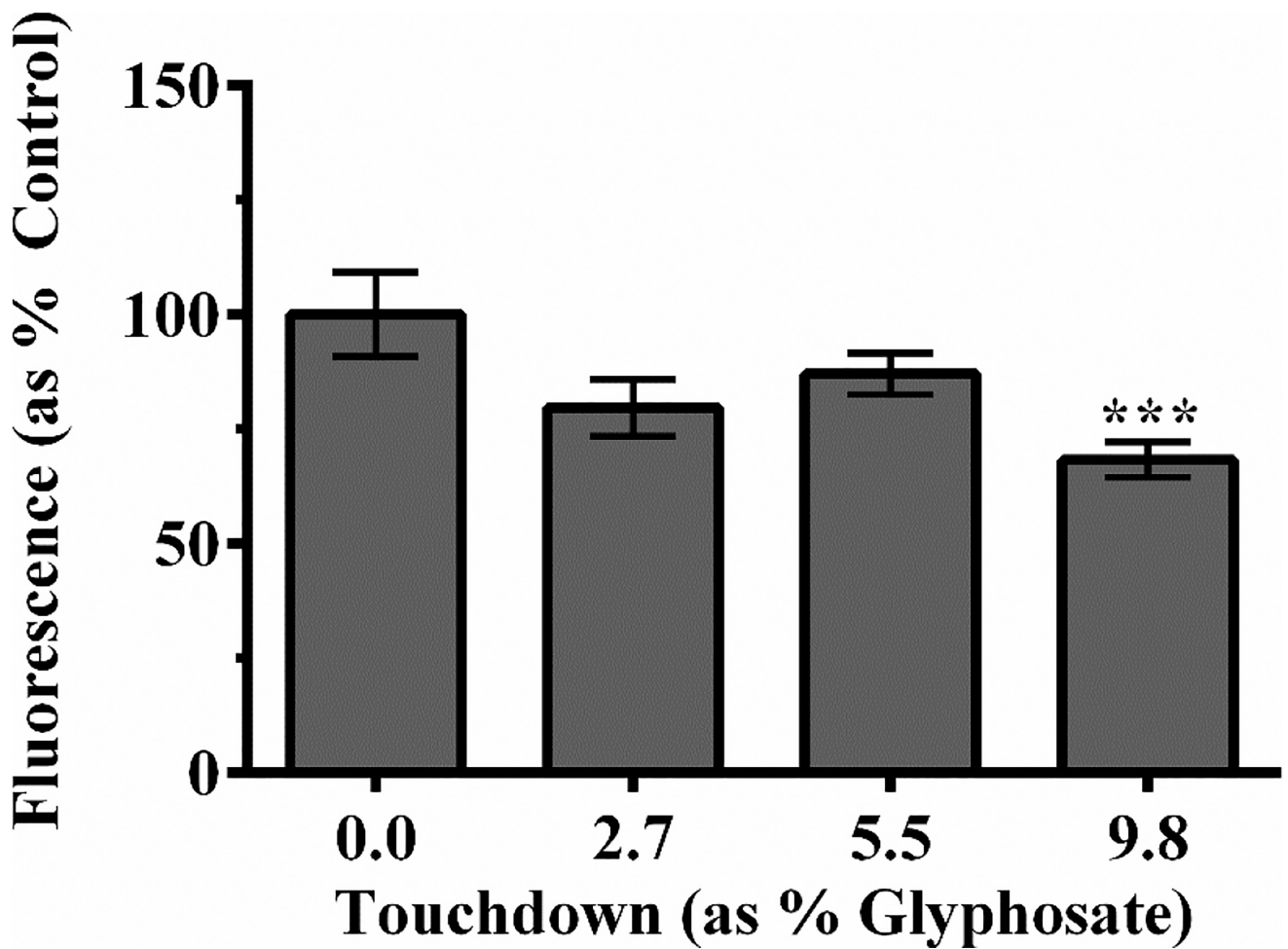


Figure 2.

Proton gradient integrity in TD-treated worms. Following treatment with TD, ANOVA indicated a statistically significant decrease in number of red pixels associated with worms in the highest treatment group. Although not statistically significant, a trend ($p = 0.0625$) towards a decrease in fluorescence was observed in the 2.7% group. Data are presented as mean pixel number \pm SEM and represent $N = 3$ separate synchronizations. *** $p < 0.001$ compared to control.

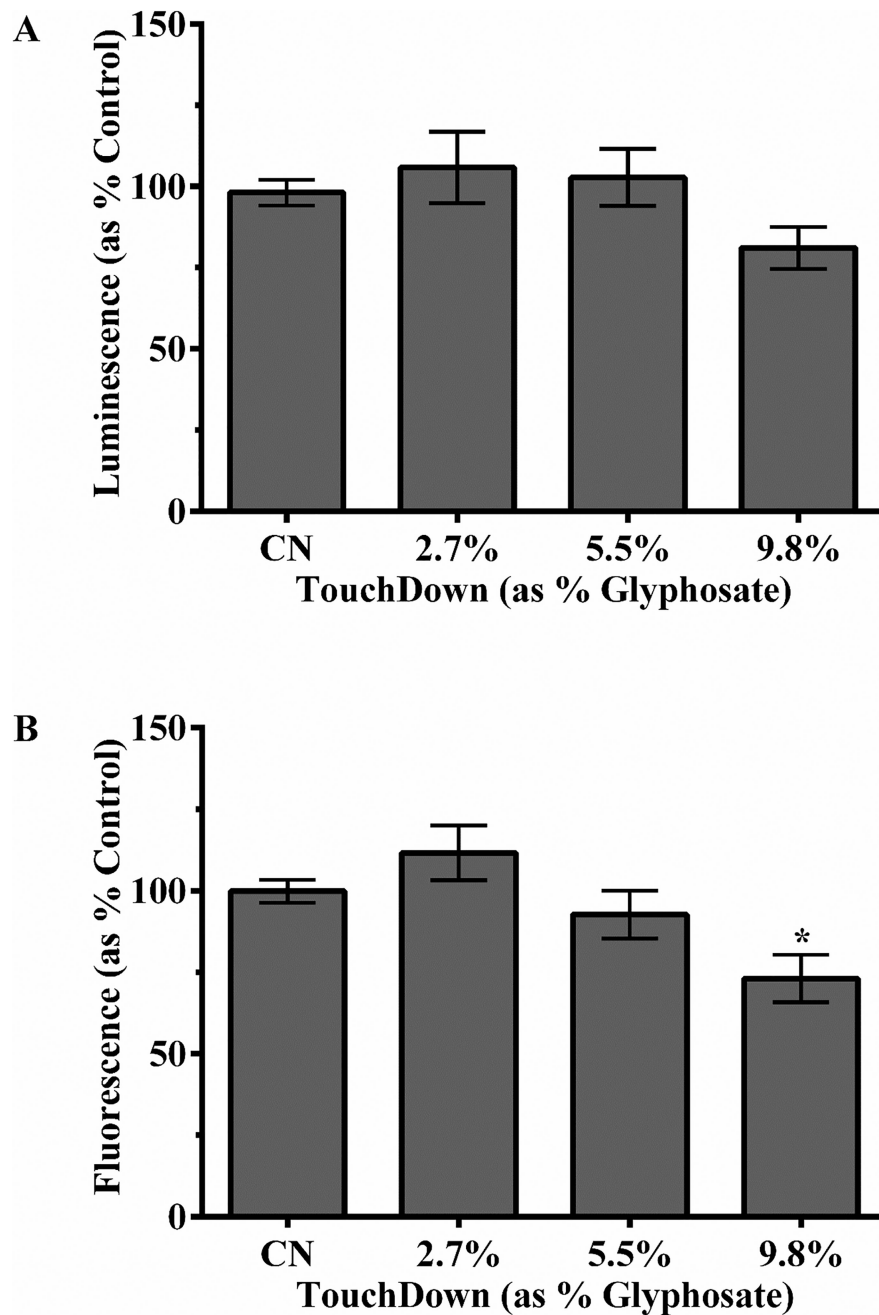


Figure 3. Relative ATP amount following TD treatment of worms. One-way ANOVA indicated no statistically significant decrease in luminescence (A), a measure of ATP, even though viability in the same group was statistically significantly lower (B). Data are presented as mean intensity \pm SEM and represent N = 3 separate synchronizations. * $p < 0.05$ compared to control worms.

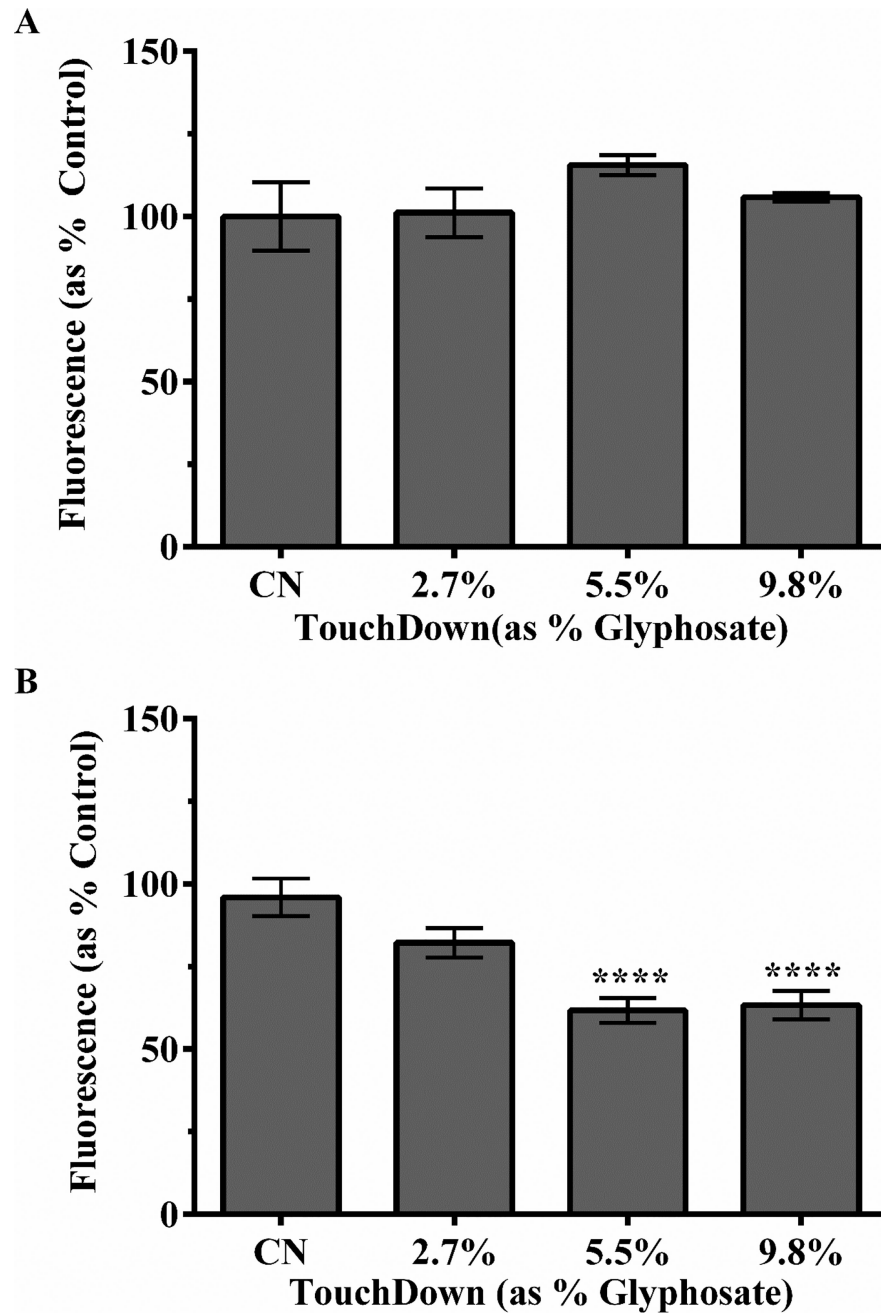


Figure 4. Use of fluorescence to assess ROS production following TD treatment of worms. In order to assess whether TD exposure could lead to increased oxidative stress, worms were exposed to DHE, to assess superoxide production (A). No statistically significant changes in DHE fluorescence was observed in groups compared to control. Chronic treatment with 5.5% or 9.8% TD resulted in a statistically significant decrease in fluorescence of AmplexRed, a probe used to detect H_2O_2 (B). Data are presented as mean intensity \pm SEM and represent N 3 separate synchronizations. **** $p < 0.001$ compared to control.

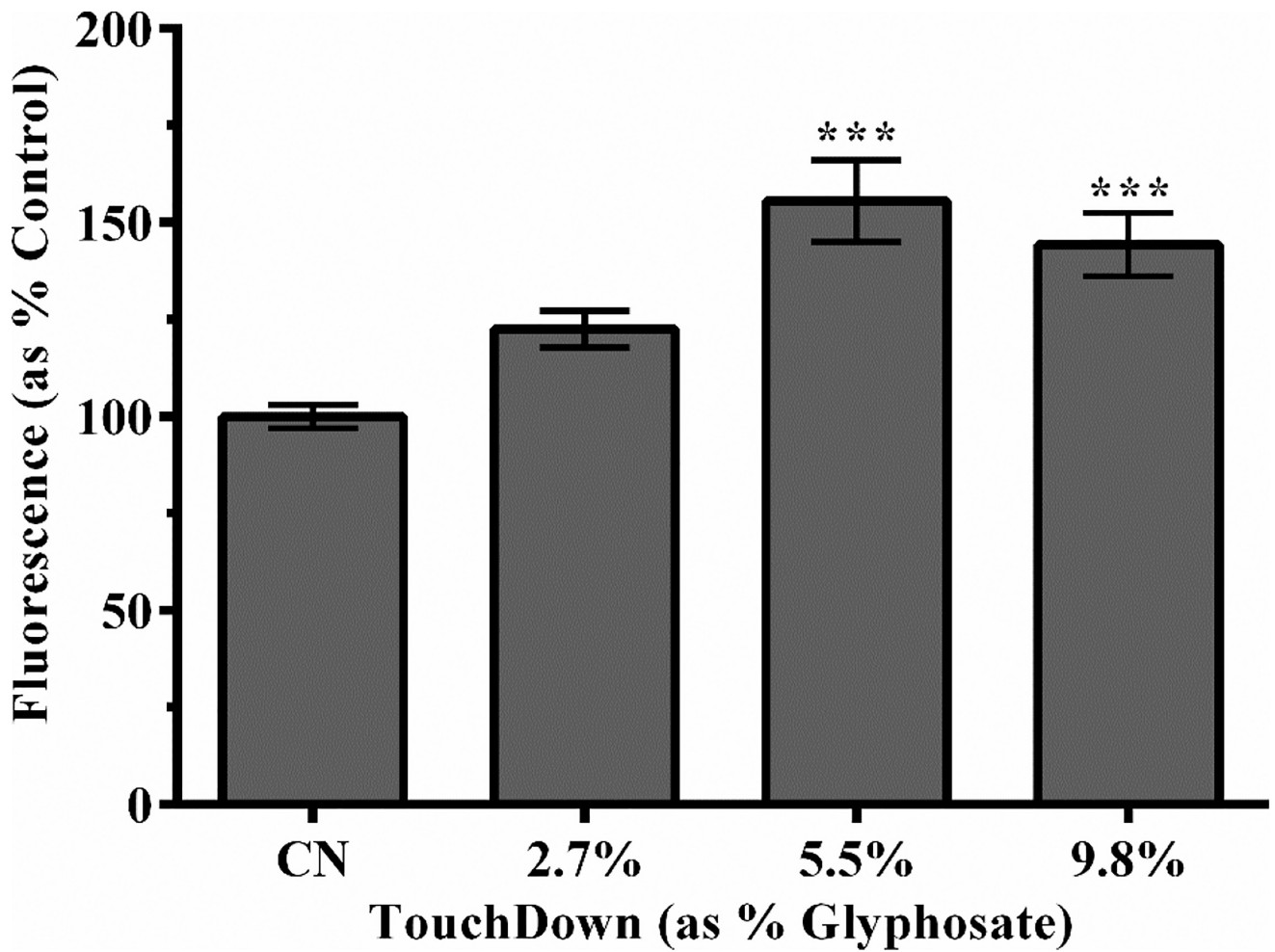


Figure 5. Increased GST-4::GFP fluorescence following treatment with TD. Transgenic worms treated with the mid- and high-level concentrations of TD showed statistically significant increases in green fluorescence compared to control worms. Data are presented as mean intensity \pm SEM and represent N = 4 separate synchronizations. ***p < 0.001 compared to controls.