

The Vitamin E analog Trolox reduces copper toxicity in the annelid *Lumbriculus variegatus* but is also toxic on its own

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Abstract

The ability of the water-soluble Vitamin E analog, Trolox, to prevent the toxic effects of copper exposure on the behavior and neuronal physiology of the freshwater oligochaete *Lumbriculus variegatus* was examined. Trolox produced a concentration-dependent increase in the 24 h LC₅₀ for copper exposure, with 100 μ M Trolox elevating the LC₅₀ by almost seven-fold (from 0.36 to 2.43 μ M). Copper exposure (0.2 μ M) for 24 h produced a reduction in the conduction velocity of the medial and lateral giant nerve fibers, which was prevented by 100 μ M Trolox. Copper exposure (0.2 μ M) for 24 h also reduced the effectiveness of substrate vibration in eliciting giant nerve fiber spikes. Trolox prevented this reduction in sensory responsiveness. Trolox (100 μ M) partially reversed the copper-induced (0.4 μ M) decrease in touch-evoked helical swimming behavior, but had no effect on the copper-induced decrement in touch-evoked body reversal. Copper exposure (0.2 μ M) for 24 h reduced the amount of spontaneous locomotion (crawling); however, Trolox did not reverse this effect. However, Trolox exposure alone produced a decrease in the distance crawled that was similar in magnitude to copper exposure. In normal worms, rapid spiking activity of the medial giant nerve fiber produces facilitation in the amplitude of the resulting muscle potentials produced by the longitudinal body wall muscles. Copper exposure had no effect on the amount of muscle potential facilitation, but Trolox exposure (100 μ M) produced a significant decrease in facilitation. The results of this study indicate that many of the toxic effects of copper exposure on *Lumbriculus* are prevented or reduced by the antioxidant Trolox. However, the results of this study also indicate that Trolox has toxic effects on behavior and neuronal physiology. The results presented here document one of the few published reports of the detrimental effects of Vitamin E or its analogs on nervous system function or behavior.

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1. Introduction

Copper is an essential element; however, it is toxic when present in excess. Copper-induced toxicity is often the result of oxidative stress; however, copper can also alter the function of enzymes, ion channels and neurotransmitter receptors (Barceloux, 1999). In humans, chronic copper toxicity is considered rare except in the case of a few diseases, such as Wilson’s disease, where copper homeostasis is impaired. However, there is a growing realization that copper-induced toxicity plays a role in many pathological conditions where derangements of

copper homeostasis are not considered the primary cause. Elevated levels of copper occur within the nervous system in conditions, such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, spongiform encephalopathies and diabetes (Rotilio et al., 2000; Campbell et al., 2001; Tapiero et al., 2003; Eaton and Qian, 2002).

The use of antioxidants, such as Vitamins C and E to reduce the effects of oxidative stress has received considerable research and clinical attention. However, in many situations, the results have been disappointing (Marchioli et al., 2001; Morley and Trainor, 2001). In both human and animal models, the effects of Vitamin E can be dose dependent, with low doses producing benefit, while higher doses produce harm (Driver and Georgeou, 2003; Miller et al., 2005).

Lumbriculus variegatus is a small freshwater oligochaete worm that lives in the shallows of freshwater ponds, lakes and marshes. There exists a large literature concerning the effects of

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toxicants on *Lumbriculus*, including several reports that specifically deal with copper exposure (Schubauer-Berigan et al., 1993; Phipps et al., 1995; Chapman et al., 1999; Veltz-Balatre, 2000; Veltz-Balatre et al., 2000; Meyer et al., 2002; Dawson et al., 2003; O'Gara et al., 2004).

In its natural habitat, *Lumbriculus* normally positions itself with its head buried in the substrate and its tail extended into the water column. The positioning of the tail into the water column renders this portion of the animal vulnerable to predation. To protect the tail, *Lumbriculus* has developed rapid withdrawal behaviors that are elicited by tactile stimulation or shadow (Zoran and Drewes, 1987; Drewes and Fournier, 1989). The neuronal circuits mediating rapid withdrawal responses in *Lumbriculus* are similar in many ways to those in other terrestrial and aquatic oligochaetes (Drewes, 1984; Zoran and Drewes, 1987). Two distinct reflex systems respond to tactile stimulation of the anterior and posterior regions of the body, respectively. Tactile stimulation of approximately the anterior one-third of the worm's body activates the medial giant fiber (MGF), while stimulation of the posterior three-fourths of the worm's body activates the paired lateral giant fibers (LGF). When activated, the giant fibers subsequently activate motor neurons, which in turn activate longitudinal body wall muscles, which underlie the rapid shortening response of the animal. An important experimental advantage of oligochaete giant fiber-mediated escape systems in toxicology research is that the electrical activity of single identified neurons can be recorded via noninvasive techniques. Because dissection is unnecessary, the physiology of these individual neurons can be repeatedly assayed from the same animal, for example, before and after a toxicant treatment.

Lumbriculus also possesses several context-specific locomotor "escape" behaviors that are not mediated by the giant fibers. In open underwater spaces, touching the head elicits a reversal in body position and touching the tail elicits helical swimming (Drewes, 1999). Helical swimming consists a series of helical waves that progress from the anterior end to the posterior end, thus propelling the worm through the water. All of these locomotor behaviors (rapid withdrawal, body reversal, helical swimming) are stereotyped behaviors that are amenable to quantification; and thus, can be used for sublethal toxicology testing (Rogge and Drewes, 1993; Ding et al., 2001; O'Gara et al., 2004).

In this report, we confirm and extend our previous observations (O'Gara et al., 2004) of the toxic effects of copper exposure on the behavior and neuronal physiology of *Lumbriculus*. In addition, we show that Trolox, a water-soluble analog of Vitamin E, was able to prevent or reduce some of the copper-induced toxic effects. However, we also document that Trolox exposure alone produced toxic effects on the behavior and neuromuscular physiology of *Lumbriculus*.

2. Methods and materials

2.1. Animal culture

L. variegatus were laboratory-reared in aquaria containing aerated artificial pond water with pieces of brown paper towels

to act as a substrate. Worms were fed commercial fish food one to two times per week (O.S.I. Pond Pellets, O.S.I. Marine Lab, Hayward, CA). The asexually reproducing cultures have been maintained in the laboratory for approximately 3 years and were derived from animals originally purchased from Aquatic Foods (Fresno, CA). The soft artificial pond water had the following composition: 1 mM NaCl, 13 μ M KCl, 4 μ M Ca(NO₃), 17 μ M Mg(SO₄), 71 μ M HEPES buffer with a pH of 7.0 ± 0.05 . No attempt was made to monitor or adjust the pH once the worms were placed in the water. Water used for the artificial pond water and all other solutions was produced by a Barnstead NANOpure system, using house RO water as the source water.

Worms used in these experiments were randomly chosen and were 2–5 cm in length as well as lacking any obvious morphological defects.

2.2. Determination of LC₅₀s

To determine the LC₅₀ to copper exposure, worms (10 per concentration) were exposed to one of six concentrations of CuSO₄ for 24 h (nominal concentrations of 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 μ M). Each worm was placed into a separate 100 mm \times 15 mm Petri dish containing 40 ml of copper-containing water. To determine the effects of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Calbiochem, La Jolla, CA) on copper toxicity, worms were placed in artificial pond water containing copper, in the concentrations mentioned above, along with Trolox at a concentration of 10, 50 or 100 μ M. Determination of lethality was made after 24 h; death was indicated by decomposition of the worm. Because *Lumbriculus* decays very rapidly following death, decomposition is a very sensitive indicator of death. We have noted that the structure of the worm can change from almost normal to complete disintegration (only the outside cuticle remains) within 2 h. Immobility (including lack of heartbeat) or lack of responsiveness to touch does not reliably indicate death since worms can sometimes completely recover from toxicant exposure once placed in clean water. The mean lethal concentration (LC₅₀) and 95% confidence interval (95% CI) were calculated by the trimmed Spearman–Karber method (Hamilton et al., 1977) using a computer program obtained from the United States Environmental Protection Agency.

2.3. Behavioral testing

2.3.1. Assessment of touch-evoked helical swimming and body reversal behaviors

We assessed the effect of various treatments on the ability of touch to evoke stereotyped helical swimming and body reversal behaviors. These behaviors were studied in a manner similar to previous studies (Drewes, 1999; Ding et al., 2001; O'Gara et al., 2004). In normal worms, touching the head evokes body reversal while touching the tail evokes helical swimming. Individual worms were placed into a 100 mm \times 15 mm Petri dish filled with 40 ml of artificial pond water or artificial pond water containing either copper sulfate (0.2 or 0.4 μ M), Trolox

(50 or 100 μM) or copper sulfate and Trolox ($N = 10$ per treatment). Testing of swimming and reversal behaviors occurred in this same dish. The experimenter assessing the behaviors was blind to each animal's treatment. Each worm was exposed to its test solution for 8 h and tested for swimming and reversal behaviors at 0 (prior to exposure), 1, 3 and 8 h of exposure. Copper concentrations and exposure times used in this and subsequent experiments in this report are based upon our previous results describing the effects of copper exposure on behavior and giant fiber physiology (O'Gara et al., 2004).

At each testing time, the worm was touched 10 times alternatively at its anterior or posterior ends (five touches per end). The interval between successive touches was 2–6 s. The tactile stimulus was delivered with a rubber probe (made from a rubber band) mounted at the end of a wooden applicator stick. The dimensions of the rubber probe were $2\text{ mm} \times 1\text{ mm} \times 7\text{ mm}$ long. A response was scored as successful only when the worm showed stereotypical helical swimming or body reversal movements (Drewes, 1999). Data are reported as the percentage of trials in which stereotypical swimming or reversal movements were successfully evoked.

2.3.2. Spontaneous crawling

Worms ($N = 10$ per treatment) were exposed for 24 h to either clean pond water, 0.2 μM copper, 100 μM Trolox or 0.2 μM copper and 100 μM Trolox. The test chamber was the top of a 60 mm plastic Petri dish containing a 55 mm diameter piece of Whatman #2 filter paper wetted with 2 ml of clean artificial pond water. A $4.8\text{ cm} \times 0.8\text{ cm}$ smooth acrylic strip with rounded ends was placed in the center of the test chamber. Due to the natural thigmotaxis of worms, they spent most of their time in the test chamber in contact with the acrylic strip, but they were free to crawl around the rest of the chamber. Each worm was placed near the acrylic strip and allowed to acclimate to the chamber for 2.5 min. To assess spontaneous crawling, a group of four worms (each in a separate chamber) was videotaped for 2.5 min using a digital video camera (JVC GR-290; Wayne, NJ). During playback, the zoom feature of the camera was used to project each chamber onto a television monitor so that it had a diameter of 200 mm. To quantify the distance crawled during the trial, the worm's head was followed around the monitor screen using a digital map measurer (Brunton MM10602; Riverton, WY). A piece of transparency film for an ink jet printer (which has a rough surface) was taped onto the monitor screen to provide a surface with adequate traction to turn the map measurer's measuring wheel. The map measurer was calibrated to display the distance traveled by the worm in millimetres.

2.4. Electrophysiological testing

Techniques for noninvasive electrophysiological testing of giant nerve fiber function in oligochaetes have been previously described (Drewes, 1984; Zoran and Drewes, 1987; Rogge and Drewes, 1993). A worm was placed perpendicular to a grid of recording electrodes on a printed circuit board. The worm was held next to a smooth acrylic strip ($4.8\text{ cm} \times 0.8\text{ cm}$) by the

surface tension caused by a drop of water placed on the circuit board. A human hair (attached to a wooden applicator stick) or a rubber probe (as above) was used to apply tactile stimulation to the anterior or posterior end of the worm and evoke medial or lateral giant fiber (MGF and LGF) activity, respectively. Two pairs of recording electrodes were used to detect evoked spikes at different points along the animal's body. Signals from the electrodes were filtered and amplified by AC-coupled amplifiers (Warner Instruments DP-301, Hamden, CT), then displayed as two channels on a computer-based data acquisition system (WinDaq [Dataq Instruments, Dayton, OH] or iWorx 214 hardware with LabScribe software [CB Sciences, Dover, NH]) at a sampling rate of 20,000 (WinDaq) or 25,000 samples/s/channel (LabScribe).

2.4.1. Measurement of giant fiber conduction velocity

Worms were tested for their MGF and LGF conduction velocities and then placed in one of three treatment solutions (100 μM Trolox [$N = 10$], 0.2 μM copper [$N = 15$] or 0.2 μM copper and 100 μM Trolox [$N = 15$]) for 24 h. At the end of the 24 h exposure time, the conduction velocity of each worm was determined again. Giant fiber conduction velocity was determined over a 10-mm conduction distance in the mid region of the worm. To obtain conduction velocity, conduction distance was divided by conduction time, as measured by the peak-to-peak interval between the two recording channels as the spike propagated between the two recording sites. During a testing session, the mean conduction velocity of each giant fiber was calculated from three to six measurements.

2.4.2. Sensory input onto the giant fibers

To assess sensory input onto the giant fibers, we vibrated the recording electrode grid using a push type solenoid (Guardian Electric A420-067074-00; Woodstock, IL). The plunger of the solenoid, which protrudes through the body of the device, was placed in contact with the electrode grid approximately 5 cm from the recording electrodes. Depending on their spontaneous locomotion, parts of a worm could be as close as 4 cm to the solenoid plunger. The solenoid was positioned so that when the solenoid was activated (by a 150 V, 250 ms pulse from a Grass S88 stimulator), the plunger lifted 3.25 mm above the electrode grid. Upon termination of the activating pulse, the plunger fell back onto the grid propelled by the force of gravity. The impact of the plunger upon the electrode grid caused vibrations that elicited giant fiber spikes. For the remainder of this report, we will refer to the plunger impact as a *tap*. To estimate the force of the tap upon the electrode grid, we positioned the solenoid above a force transducer (CB Sciences FT-100, Dover NH) and noted the maximum force generated by 10 taps. The average maximal tap force was $206.86 \pm 10.03\text{ mN}$ (S.E.M.).

Worms ($N = 10$ per treatment) were exposed for 24 h to control water, 0.2 μM copper, 100 μM Trolox or 0.2 μM copper and 100 μM Trolox. During a test session, a worm was placed upon the recording grid next to the acrylic strip and was not touched for the remainder of the test. The worm was allowed to spontaneously crawl around the acrylic strip and a

tap was initiated when the worm was centered over the two pairs of recording electrodes. Almost all tap-evoked giant fiber spikes from control worms were MGF spikes regardless of the animal's orientation on the recording grid (i.e., which end of the worm was closer to the solenoid). A test session consisted of 10 taps. The animal's score for the session was the number of trials (out of 10) that produced at least one tap-evoked giant fiber spike (either an MGF or a LGF spike).

2.4.3. Measurement of muscle potential facilitation

During noninvasive recordings of giant fiber function from oligochaetes, it is also possible to record longitudinal muscle potentials evoked by the giant fibers (via their associated motor neurons) (Drewes, 1984). Each MGF spike in *Lumbriculus* (and other oligochaetes) produces an associated muscle potential (Fig. 7A). In both terrestrial earthworms and *Lumbriculus*, rapid firing of the MGF (and associated motor neurons) produces facilitation in the amplitude of the muscle potentials (Günther, 1972; Drewes et al., 1980; O'Gara et al., 1982; Zoran and Drewes, 1987; Fig. 7A). In the earthworm *Lumbricus terrestris*, the degree of muscle potential facilitation is dependent on the MGF interspike interval, with shorter interspike intervals producing greater facilitation (Drewes et al., 1980). Drewes et al. computed muscle potential facilitation ratios by comparing the amplitude of noninvasively recorded muscle potentials associated with two closely spaced MGF spikes. It is difficult to calculate muscle potential ratios in the same way in *Lumbriculus* for two reasons: (1) the amplitude of the muscle potential associated with the first MGF spike is smaller and more variable than in terrestrial earthworms and (2) in *Lumbriculus*, the MGF can spike at a faster rate than in the terrestrial earthworms. The result of this more rapid spiking is that the first muscle potential can sum with the second MGF spike; thus, distorting the amplitude (and waveform) of each potential. To get around these difficulties, we used the amplitude of the initial MGF spike as the reference amplitude (rather than the muscle potential associated with the first MGF spike) for calculating the amount of facilitation of the second muscle potential. The details on how muscle potential facilitation ratios were calculated are presented in Fig. 7A. Because the amount of muscle potential facilitation varies along the length of the worm (Drewes et al., 1980; Zoran and Drewes, 1987), we measured facilitation ratios in recordings made from the anterior one-fourth of the worm. Worms were exposed to control water, 0.2 μM copper or 100 μM Trolox for 24 h. Each worm was placed on the recording grid and touched with varying intensities by a hand-controlled probe (as above). By varying the intensity of the touch, different medial giant fiber interspike intervals were obtained. Control data consisted of 152 recordings collected from 35 worms, data from copper-treated worms consisted of 59 recordings collected from 21 worms and data from Trolox-treated worms consisted of 178 recordings from 20 worms. Data was grouped into bins depending on the MGF interspike interval (1–2, 2–3 ms, etc.). The number of data points in any one bin ranged from 5 to 45 recordings (mean = 21.44 ± 12.95 S.D.).

2.5. Data analysis

Except for the determination of the LC_{50} to copper exposure (described above), all statistics were performed using Sigma-Stat 2.03 (Systat Software, Port Richmond, CA). Results from statistical tests were considered significant if $p < 0.05$. Data are usually reported as means \pm standard error of the mean (S.E.M.). When data were not normally distributed, they are reported as median \pm median absolute deviation (MAD).

3. Results

3.1. Effects of Trolox on the LC_{50} for copper exposure

If the toxic effects of copper exposure on *Lumbriculus* are at least partially due to the copper-induced formation of reactive oxygen species, then simultaneous treatment with an antioxidant should decrease the levels of reactive oxygen species in the animal; and thus, reduce copper toxicity. Simultaneous exposure to copper and Trolox increased the LC_{50} over the LC_{50} to copper exposure alone (Fig. 1). Based upon the lack of overlap of the 95% confidence intervals, both 50 and 100 μM Trolox elevated the LC_{50} for copper exposure, but the 10 μM concentration did not (Fig. 1B). Simultaneous exposure to 100 μM Trolox and copper elevated the LC_{50} (2.43 μM ; 95% CI 1.84–3.19 μM) by a factor of almost seven-fold over the LC_{50} for copper exposure alone (0.36 μM ; 95% CI 0.22–0.60 μM).

3.2. Effects of Trolox on copper-induced reductions in touch-evoked body reversal and helical swimming activity

In our previous study (O'Gara et al., 2004), copper exposure reduced the ability of touch to successfully evoke body reversal and helical swimming behaviors. In this study, exposure to 0.4 μM copper induced significant reductions in touch-evoked body reversal and helical swimming within 1 h of initial exposure (Fig. 2). Trolox (100 μM) was ineffective in protecting the worms from copper-induced reductions in evoked body reversal behaviors (Fig. 2A). In contrast, 100 μM Trolox significantly reduced the severity of the copper-induced reductions in evoked helical swimming behavior at 1 and 3 h after initial exposure, but not at 8 h of exposure.

Similar to our previous results (O'Gara et al., 2004), exposure to 0.2 μM copper alone produced smaller deficits in both body reversal and helical swimming behaviors than was produced by 0.4 μM copper (not shown). Trolox provided no protection from the deficits produced in these two behaviors by 0.2 μM copper (two-way repeated measures ANOVA with Student–Newman–Keuls post hoc comparisons between cell means).

3.3. Trolox-induced reductions in touch-evoked body reversal and swimming activity

As one of the controls for the above experiments on evoked body reversal and helical swimming, we also exposed worms to 50 and 100 μM Trolox alone. Trolox alone produced

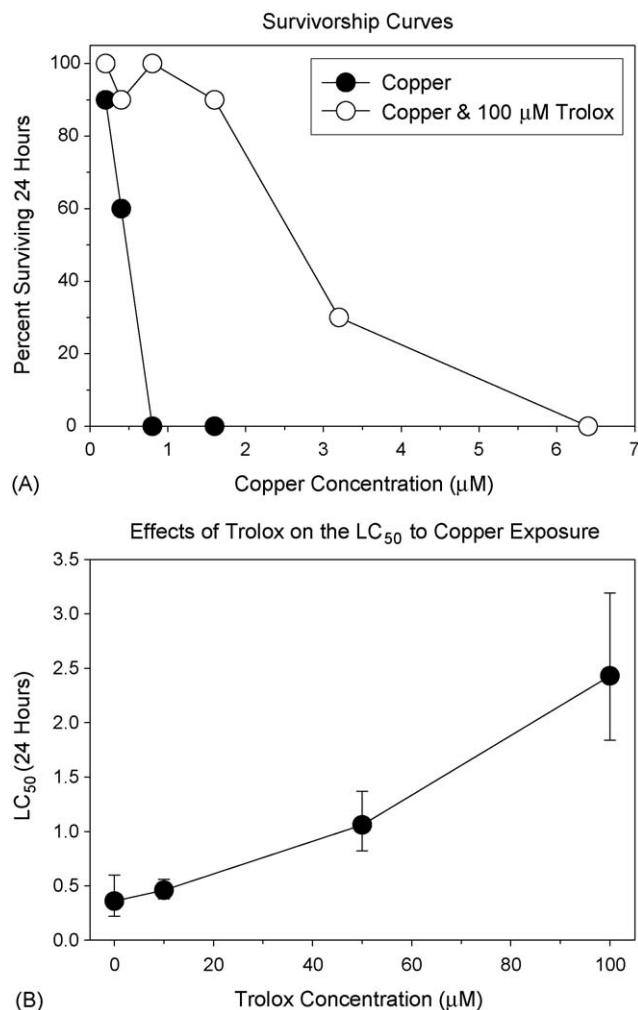


Fig. 1. Trolox elevated the LC₅₀ for copper exposure. (A) Survivorship curves at 24 h for animals exposed to several copper concentrations or copper and 100 μM Trolox. Survivorship curves were also generated for 10 and 50 μM Trolox (and copper), but are not plotted for clarity. (B) Trolox produced a concentration-dependent increase in the LC₅₀ for copper exposure. Whisker bars indicate the 95% confidence intervals.

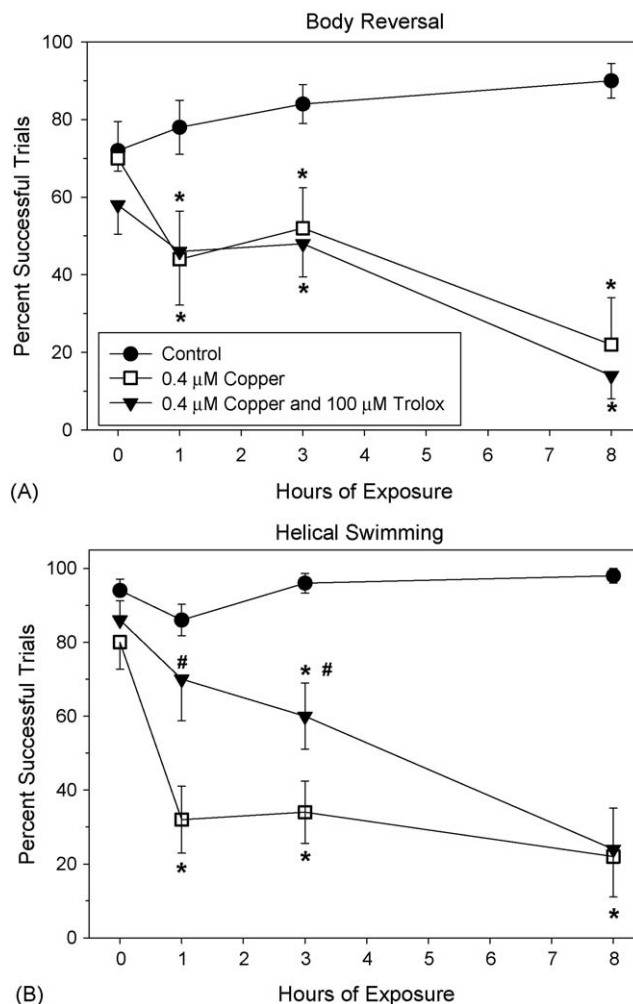


Fig. 2. Effects of Trolox on copper-induced deficits in touch-evoked body reversal and helical swimming behaviors. (A) Exposure to copper reduced the ability of tactile stimulation to elicit body reversal at 1, 3 and 8 h of exposure. Trolox did not protect the animals from the copper-induced deficit. A two-way repeated measures ANOVA indicated a significant effect of treatment ($p < 0.001$; $F = 14.797$; d.f. = 2,27), duration of exposure ($p < 0.001$; $F = 6.644$; d.f. = 3,81) and the interaction ($p < 0.001$; $F = 4.753$; d.f. = 6,81). Subsequent Student–Newman–Keuls tests indicated that copper-exposed as well as copper- and Trolox-exposed animals exhibited a significant reduction in reversal behavior at 1, 3 and 8 h of exposure (*). There were not significant differences between the copper-exposed or the copper- and Trolox-exposed animals at any time point. (B) Copper exposure reduced the ability of tactile stimulation to elicit helical swimming behavior at 1, 3 and 8 h of exposure. A two-way repeated measures ANOVA indicated a significant effect of treatment ($p < 0.001$; $F = 29.687$; d.f. = 2,27), duration of exposure ($p < 0.001$; $F = 15.496$; d.f. = 3,81) and the interaction ($p < 0.001$; $F = 6.278$; d.f. = 6,81). Subsequent Student–Newman–Keuls tests indicated that compared to controls, copper produced a significant reduction in the ability of tactile stimulation to elicit helical swimming at 1, 3 and 8 h of exposure. At 1 h of exposure, the copper and Trolox-treated animals were not significantly different from controls, but were different from the copper-treated animals. By 3 h, the copper and Trolox-treated animals showed a significant decrease relative to controls, but showed a smaller decrement than copper-treated animals. An asterisk (*) indicates that the identified group was significantly different from the control group at the time point. A number sign (#) indicates that the copper and Trolox-exposed animals were significantly different from the copper-exposed animals at the time point.

concentration-dependent reductions in the ability of touch to evoke either body reversal or helical swimming behaviors (Fig. 3). Exposure to 100 μM Trolox for as little as 3 h produced significant reductions in both evoked body reversal and helical swimming. Exposure to 50 μM Trolox for as little as 3 h reduced the ability of touch to evoke helical swimming, but did not statistically reduce the ability to evoke body reversal until 8 h of exposure.

3.4. Effects of copper and Trolox on spontaneous crawling

In our previous report (O'Gara et al., 2004), we noted in an anecdotal fashion that copper exposure-induced lethargy. To quantify this lethargy, we videotaped the spontaneous locomotion of copper- and Trolox-exposed worms and determined the distanced crawled over a 2.5 min test session. We used a copper concentration of 0.2 μM because worms can live in this concentration for at least a week (i.e., no deaths

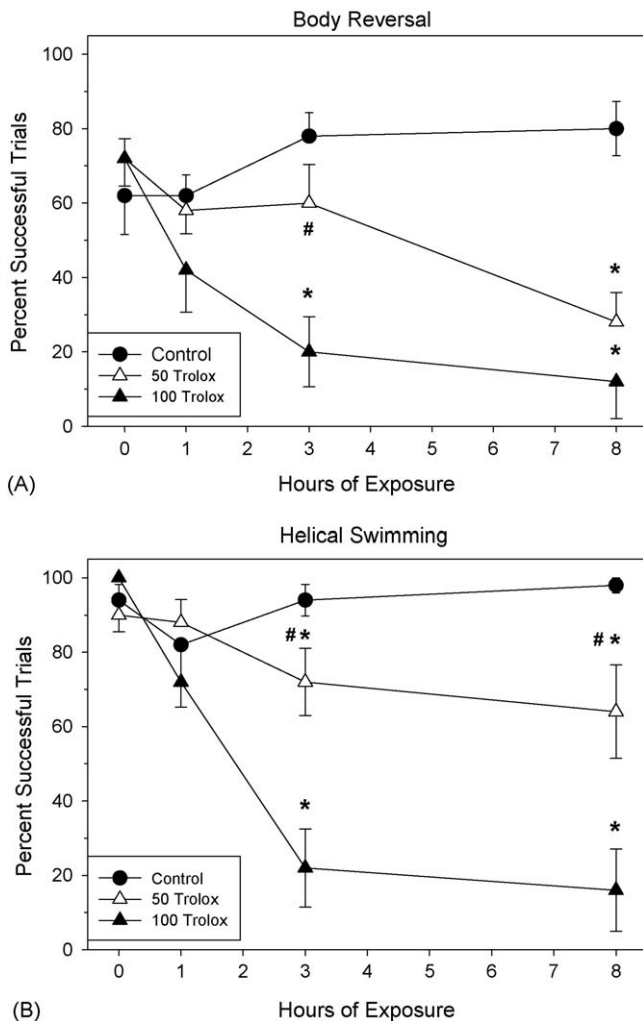


Fig. 3. Trolox exposure produced significant deficits in the ability of tactile stimulation to evoke body reversal (A) or swimming behaviors (B). In both panels, the control data is replotted from Fig. 2. (A) Exposure to 100 μM Trolox produced a significant reduction in touch-evoked body reversal behavior at 3 and 8 h of exposure, while 50 μM Trolox only produced a significant reduction at 8 h. A two-way repeated measures ANOVA indicated a significant effect of treatment ($p < 0.001$; $F = 15.325$; d.f. = 2,27), duration of treatment ($p < 0.001$; $F = 6.008$; d.f. = 3,81) and the interaction ($p < 0.001$; $F = 5.499$; d.f. = 6,81). In this panel, and in panel B, an asterisk (*) indicates that the data point is different from control at that time point and a number sign (#) indicates that the 50 μM Trolox group is significantly different from the 100 μM Trolox group at that time point. (B) Exposure to either Trolox concentration produced a significant reduction in touch-evoked helical swimming at 3 and 8 h of exposure. At these time points, exposure to 100 μM Trolox produced a significantly greater effect than exposure to 50 μM Trolox. A two-way repeated measures ANOVA indicated a significant effect of treatment ($p < 0.001$; $F = 14.960$; d.f. = 2,27), treatment duration ($p < 0.001$; $F = 18.951$; d.f. = 3,81) and the interaction ($p < 0.001$; $F = 11.918$; d.f. = 6,81).

occur). Exposure to 0.2 μM copper for 24 h reduced spontaneous locomotion to about one-third of the distance crawled by control worms (Fig. 4). To determine if Trolox could protect the worms from this copper-induced lethargy, we exposed worms to Trolox or Trolox and copper. Exposure to 100 μM Trolox alone for 24 h reduced the distance crawled by a similar magnitude to copper exposure alone. Exposure to copper and Trolox (0.2 and 100 μM , respectively) for 24 h

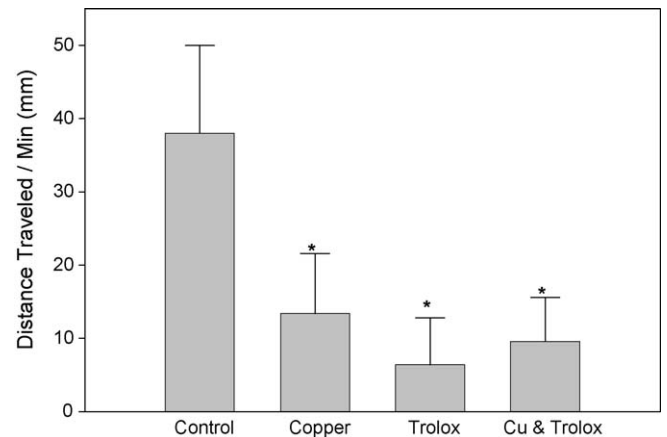


Fig. 4. Both copper and Trolox exposure reduced spontaneous crawling. The distance crawled during a trial is expressed as the number of millimetres crawled per minute. Because the data was not normally distributed, it is plotted as the median \pm the MAD. A Kruskal–Wallace one-way ANOVA indicated the treatments had a significant effect on distance crawled ($p = 0.001$; $H = 16.196$; d.f. = 3). Subsequent Student–Newman–Keuls tests indicated that each of the three treatments produced a significant decrease in distance crawled (*) compared to controls. However, the three treatment groups were not significantly different from one another.

produced a similar reduction in distance crawled as copper exposure alone or Trolox alone. Thus, not only did Trolox fail to protect the worms from copper-induced lethargy, Trolox by itself produced lethargy.

We also noted copper- and Trolox-induced lethargy while performing noninvasive electrophysiological tests of giant fiber function (below). However, we noted some qualitative differences between the lethargy produced by each substance. During noninvasive electrophysiological testing, the worms are free to crawl about on the electrode grid. Although, we have not quantified the amount of crawling, normal worms were often in motion crawling around the acrylic bar on the electrode grid (the same acrylic bar was used for electrophysiological and spontaneous crawling tests). Once positioned over the electrodes, Trolox-treated worms rarely crawled, while copper-treated animals did crawl, although less so than normal worms. We also noted differences in the behavioral responses of treated worms to tactile stimulation during electrophysiological testing. In all worms, elicitation of a single giant fiber spike did not produce an observable behavioral response. When more intense tactile stimulation was applied (producing several giant fiber spikes), we noted behavioral differences between the animals in each treatment group. Normal worms often rapidly crawled away from the stimulation site and then calmed down after a few seconds. Copper-treated animals often required a more intense touch to elicit giant fiber spiking (see below for quantification), but if they did respond, the response was often very intense and qualitatively different from that in normal worms. In response to intense tactile stimulation, copper-treated worms often engaged in side-to-side thrashing, a behavior rarely seen in normal worms following similar giant fiber spiking responses. In contrast to normal and copper-treated worms, Trolox-treated worms rarely crawled from the stimulation site, even when high frequency trains of giant fiber spikes were evoked.

3.5. Trolox protects giant nerve fibers from copper-induced reductions of conduction velocity

In our previous report (O'Gara et al., 2004), exposure to 0.2 μ M copper for 24 h caused a decrease in medial giant fiber (MGF) and lateral giant fiber (LGF) conduction velocities. After replicating that finding, we sought to determine if Trolox could protect the giant fibers from copper-induced reductions in conduction velocity (Fig. 5). Simultaneous exposure to 100 μ M Trolox was able to prevent the copper-induced decreases in MGF and LGF conduction velocities. Exposure to 100 μ M

Trolox alone for 24 h had no effect on the conduction velocities of either type of giant fiber.

3.6. Effects of copper and Trolox on sensory input onto the giant nerve fibers

In this and our previous report (Fig. 2; O'Gara et al., 2004), we documented that copper exposure reduced the ability of touch to evoke body reversal and helical swimming behaviors. One potential explanation for the reduced effectiveness of touch in evoking these behaviors could be reduced effectiveness of sensory input. In terrestrial earthworms, sensory input also modulates the activity of the crawling central pattern generator (Mizutani et al., 2004). The reductions we noted in spontaneous crawling (Fig. 4) could, at least in part, be the result of reduced sensory input onto the crawling central pattern generator. Because of the small size of *Lumbriculus* (less than 1 mm in diameter), it is difficult to apply a calibrated touch directly to the animal's body surface. As an alternative strategy, we applied calibrated taps to the animal's substrate (the printed circuit board electrode grid), which would vibrate the board and evoke giant fiber spikes. To allow for detection of treatment-induced hypo- and hypersensitivity, the force of the tap was calibrated (using control animals) to produce a giant fiber spike in response to approximately one-half the taps (Fig. 6). Copper exposure (0.2 μ M for 24 h) produced a significant decrease in the number of trials (out of 10 trials) that produced at least a single giant fiber spike in response to a tap. Trolox (100 μ M) was able to protect the animal from the copper-induced decrease in responsiveness. Exposure to Trolox alone had no effect on the number of trials that produced giant fiber spikes.

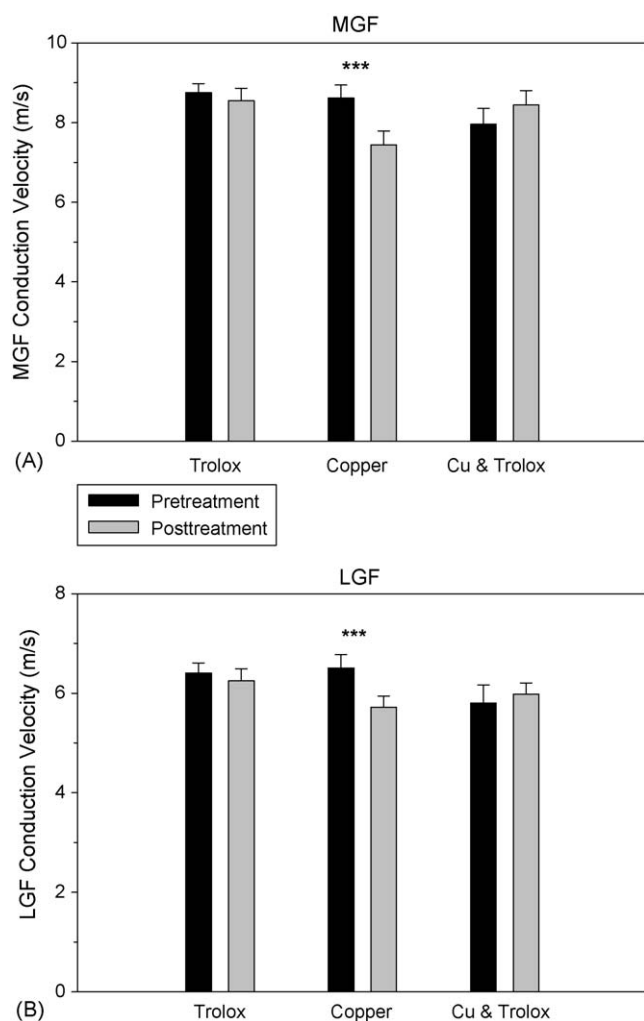


Fig. 5. Trolox prevented the copper-induced giant fiber conduction velocity decrease. For the MGF data (A), a two-way repeated measures ANOVA failed to find a significant effect of treatment ($p = 0.43$; $F = 0.864$; d.f. = 2,37), or test time (pretreatment vs. post-treatment) ($p = 0.83$; $F = 3.177$; d.f. = 1,37), but the interaction was significant ($p < 0.001$; $F = 9.760$; d.f. = 2,37). For the LGF data (B), a two-way repeated measures ANOVA failed to find a significant effect of treatment ($p = 0.531$; $F = 0.644$; d.f. = 2,37), but did find significant differences for test time (pretreatment vs. post-treatment) ($p = 0.012$; $F = 7.06$; d.f. = 1,37) and the interaction ($p < 0.001$; $F = 10.109$; d.f. = 2,37). For each giant fiber (MGF AND LGF considered separately), subsequent Tukey tests indicated that copper treatment significantly reduced ($p < 0.001$) conduction velocity (***). The lack of a significant difference between the pretreatment and post-treatment mean conduction velocities in the copper and Trolox-treated group indicates that Trolox prevented the copper-induced decrease.

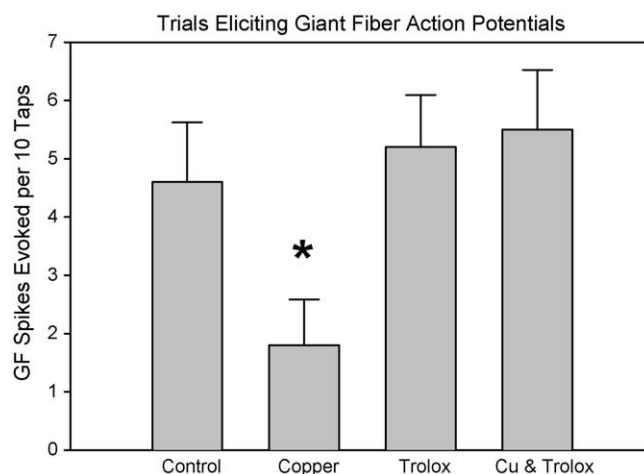


Fig. 6. Trolox prevented the copper-induced reduction in the effectiveness of sensory input onto the giant fibers. The number of trials (out of 10) in which a tap to the substrate elicited at least a single giant fiber spike is plotted in the graph. A one-way ANOVA indicated that significant differences existed among the treatments ($p = 0.033$; $F = 3.260$; d.f. = 3,36). Subsequent Student–Newman–Keuls tests indicated that animals treated with copper alone produced significantly fewer spiking trials than the other three treatments (which were not significantly different from one another).

3.7. Effects of copper and Trolox on neuromuscular function

When muscle potential facilitation ratios from *Lumbriculus* are plotted against the MGF interspike interval (Fig. 7B), the resulting graph is qualitatively similar to the comparable figure constructed from data derived from the earthworm *L.*

terrestris (Drewes et al., 1980). In this study, 24 h exposure to 100 μ M Trolox significantly reduced the amplitude of the muscle potential facilitation ratio at all MGF interspike intervals between 1 and 10 ms (Fig. 7B). Over a similar range of MGF interspike intervals, 24 h exposure to 0.2 μ M copper did not alter the facilitation ratios from those recorded from control worms.

4. Discussion

In this report, we have replicated and extended our previous findings (O'Gara et al., 2004) on the toxic effects of copper exposure on the behavior and neuronal physiology of *L. variegatus*. With respect to the giant fiber reflexes, copper exposure reduced the effectiveness of sensory input onto the giant fibers (Fig. 6) as well the giant fiber conduction velocity (Fig. 5), but did not significantly alter the output side of at least the MGF system (Fig. 7). Copper exposure also reduced spontaneous locomotion (Fig. 4) and touch-evoked escape behaviors (Fig. 2). We have also demonstrated that some of these copper-induced deficits can be reduced or prevented by the antioxidant Trolox. However, we have also documented that Trolox exhibits toxicity since it reduced spontaneous locomotion, reduced the ability of tactile stimulation to evoke nongiant mediated escape behaviors (body reversal and helical swimming), and produced abnormalities in neuromuscular physiology (Figs. 3, 4 and 7).

In an intact animal, with many interacting systems, it is almost impossible to precisely determine the site or mode of action of a toxicant. Although the current study appears to demonstrate specific toxic actions of copper and Trolox upon the nervous system, it is possible that these effects occur secondarily due to a primary site of toxicant action outside the nervous system. Thus, the effects we note here may not be due specifically to a direct action of the toxicants on the nervous system. However, the fact that some physiological parameters measured in the current study were unaltered by either copper or Trolox exposure argues against all of the noted toxic effects being due to a general decline in animal health. Another variable that may affect the mode of action of the toxicants used in this study is the duration of exposure. Toxicity due to short duration exposures may be mediated by fundamentally different mechanisms than toxicity to longer duration exposures.

4.1. Potential mechanisms of copper-induced toxicity in *Lumbriculus*

The generation of reactive oxygen species is generally recognized as one of the most important mechanisms mediating copper toxicity (Pourahmad and O'Brien, 2000; Burkitt, 2001; Gaetke and Chow, 2003). In the present study, our results are consistent with a number of the toxic actions of copper being mediated by reactive oxygen species since they can be prevented or reduced by the simultaneous application of the antioxidant Trolox. Although both copper and Trolox could produce toxicity through mechanisms unrelated to their effects

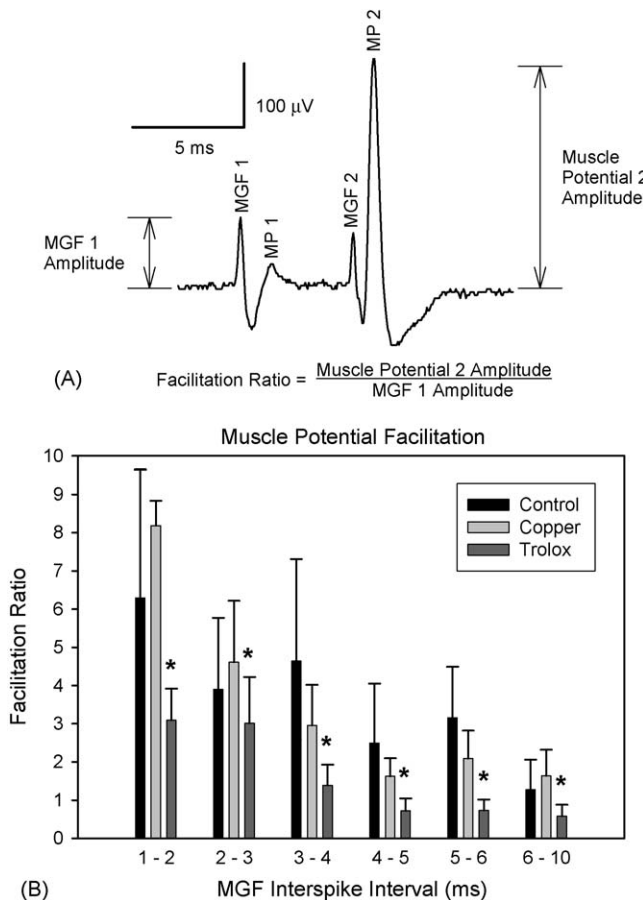


Fig. 7. Trolox reduced muscle potential facilitation caused by rapid MGF spiking. (A) Methods used to calculate the muscle potential facilitation ratio. The record shows two MGF spikes (MGF 1 and MGF 2) and their associated muscle potentials (MP 1 and MP 2). Note the much greater amplitude of MP 2 compared to MP 1; this is muscle potential facilitation. The animal was touched with sufficient force to produce an MGF interspike interval of approximately 5 ms. The muscle potential facilitation ratio was calculated by dividing the amplitude of MP 2 by the amplitude of MGF 1. In the record shown here, the facilitation ratio was approximately 3.5. (B) Muscle potential facilitation ratio plotted against MGF interspike interval. At MGF interspike intervals ranging from 1 to 10 ms, the muscle potential facilitation ratio was significantly smaller for Trolox-treated worms compared to the other two groups (*). Within this same range of interspike intervals, facilitation ratios for control and copper-treated worms did not significantly differ. Because the data was not normally distributed (except for the 6–10 ms bin), the data is plotted as the median \pm the MAD. Even though the data in the 6–10 ms bin was normally distributed, the data is plotted as the median \pm MAD to maintain consistency. The data in each bin was tested for statistical differences using a series of ANOVAs (one ANOVA per bin). A Kruskal–Wallace ANOVA on ranks was performed for all bins except the 6–10 ms bin where a parametric ANOVA was performed. The ANOVAs on all bins indicated significant differences with p -values between 0.001 and 0.003, except for the 6–10 ms bin where $p = 0.016$. Subsequent tests were performed using Dunn's Method for the Kruskal–Wallace ANOVAs and a Student–Newman–Keuls test for the parametric ANOVA.

on reactive oxygen species, we are unaware of any reports that document Trolox-mediated (or Vitamin E analog-mediated) protection from copper-induced toxicity unless the copper-induced toxicity is mediated by the formation of reactive oxygen species.

4.1.1. Conduction velocity

In this study, we demonstrated that Trolox prevented copper-induced reductions in giant fiber conduction velocity (Fig. 5). It is curious that although there are several studies showing the benefits of antioxidants in reducing the severity of liver damage in animal models of Wilson's disease (Yamazaki et al., 1993; Hawkins et al., 1995), there appear to be no controlled studies that assess the value of antioxidant therapy in ameliorating the severity of the neuropathology in Wilson's disease or the animal models of the disease.

4.1.2. Sensory systems

Copper exposure reduced the sensitivity of the *Lumbriculus* giant fibers to vibratory sensory stimuli (Fig. 6). The reduction in sensitivity to vibration induced by copper exposure was prevented by simultaneous exposure to Trolox. The experiments performed in the current study do not allow determination of the processes affected by copper exposure that produce this insensitivity. Copper exposure could alter the sensory fibers themselves, or they might alter the responsiveness of the giant fibers to sensory input. Sensory transduction could be impaired by copper exposure as occurs in other systems (Iwasaki and Sato, 1984; Baatrup, 1991; Sandahl et al., 2004). Synaptic transmission could be reduced via either presynaptic (Komulainen and Tuomisto, 1981; Salánki et al., 1993; Erdélyi et al., 1998; Wang, 1999) or postsynaptic mechanisms (Lovinger, 1991; Ma and Narahashi, 1993; Trombley et al., 1998; Horning and Trombley, 2001).

4.2. Toxicity of Trolox

In this study, Trolox reduced: (1) the ability of tactile stimulation to evoke body reversal and swimming behaviors (Fig. 3), (2) spontaneous crawling behavior (Fig. 4) and (3) the amount of muscle potential facilitation produced in response to rapid MGF spiking activity (Fig. 7). However, Trolox had no effect on giant fiber conduction velocity (Fig. 5) or the effectiveness of sensory input (Fig. 6). Because sensory input onto the giant fibers was apparently normal following Trolox treatment (Fig. 6), it seems unlikely that a generalized sensory deficit could explain the reduced ability of tactile stimulation to evoke body reversal and helical swimming behaviors or the reduction in spontaneous crawling. The giant fibers of earthworms receive input from two types of mechanoreceptors (Günther, 1970; Smith and Mittenthal, 1980). These same sensory fibers are likely to mediate touch-evoked body reversal and helical swimming. It has been suggested that at least some of the synapses of these sensory neurons onto the giant fibers are electrical, which helps to assure the rapidity and reliability of synaptic input (Smith and Mittenthal, 1980; Drewes, 1984). The fact that in normal *Lumbriculus*, tactile stimulation is not

100% effective in eliciting body reversal or helical swimming suggests that the sensory neurons make chemical synapses onto the neuronal circuits mediating these behaviors. Because sensory input onto the crawling central pattern generator is graded (Mizutani et al., 2004), these synapses are also likely to be chemical in nature. Thus, if nongiant mediated escape behaviors and spontaneous crawling were dependent on chemical synaptic input, Trolox might affect them, while the electrical synaptic input onto the giant fibers remained unaffected. The Trolox-induced reduction of muscle potential facilitation (Fig. 7) is suggestive of a direct change in the effectiveness of chemical synaptic transmission.

It is curious that for those measures where both copper and Trolox exposure each induced deficits (body reversal, helical swimming and spontaneous crawling) that the simultaneous application of both substances did not produce an even larger deficit (i.e., the deficits induced by each substance were not additive). Because we do not know the precise mechanisms producing these deficits following exposure to each substance, it is not possible to explain why the combined toxic effects are not additive. However, the phenomenon is reminiscent of situations where saturation of a common site of action occurs. For example, if one toxicant produced maximal inhibition of a system, addition of another toxicant that acted at the same site could not produce further inhibition.

Toxic effects of Vitamin E (or its analogs) are not unknown, although documented by a relatively small number of studies (Thorton et al., 1995; Bast and Haenen, 2002; Cornwell et al., 2002; Rietjens et al., 2002; Calviello et al., 2003; Driver and Georgeou, 2003; Miller et al., 2005). At least under some conditions, Trolox can act as a prooxidant rather than as an antioxidant (Ko et al., 1994; Burkitt and Milne, 1996; Gyulkhanyan et al., 2003; Diaz et al., 2005). Without a direct assay of reactive oxygen species in *Lumbriculus*, it is impossible to exclude the possibility that Trolox produces its toxic effects via a prooxidant action. However, if both copper and Trolox were acting as prooxidants, they would be expected to produce similar deficits. While both copper and Trolox produced deficits in the ability of tactile stimulation to elicit reversal and swimming behaviors as well as reduced spontaneous locomotion, there were several measures where the two substances produced different effects. Trolox did not exhibit toxicity, and was able to protect the worms from the toxic effects of copper in a number of assays (LC₅₀, giant fiber conduction velocity and sensitivity to vibration); but in contrast, Trolox reduced muscle potential facilitation which was unaffected by copper exposure. When considering the reduced locomotion induced by copper or Trolox (Fig. 4), the lethargy induced by copper was qualitatively different than that induced by Trolox. These differential effects of copper and Trolox suggest different modes of action for the two substances in causing at least some aspects of their respective toxicities.

Copper- and Trolox-induced toxicity may also differ because of the differential solubility of the two substances, with Trolox being more lipid soluble than copper. This may lead to differential distribution of the two substances in either body or cellular compartments. In addition, the presence of

differential transport mechanisms for copper and Trolox also could lead to differential distribution within the body or cells. Thus, even if both toxicants had the same mechanism of action, their differential distribution might lead to different symptomatology.

If Trolox-induced toxicity in *Lumbriculus* is not the result of a prooxidant effect of Trolox, what mechanisms actually produce the toxicity? Although often under appreciated, Vitamin E and its analogs are known to have many actions that are not dependent on their antioxidant properties (Bast and Haenen, 2002; Zingg and Azzi, 2004). These effects are wide ranging and include specific interactions with receptors, enzymes, structural proteins, lipids and transcription factors. In this report, we have developed a number of sensitive measures that quantify the toxic effects of Trolox. With this knowledge, we can now use *Lumbriculus* behavior and physiology to determine if the toxic effects of Trolox are due to its potential prooxidant actions or other mechanisms that are divorced from oxidative stress.

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