The Effect of Vitamin E on the Survival Rate of *unc-13* *Caenorhabditis elegans* mutants under Oxidative Stress

Jessica Porcelan[1], Erica Schindewolf[1], Kaitlyn Storey[1], Kirsten King[1], Kari Hart[1], Rebecca Kohn[1]
[1]*Ursinus College, Collegeville PA 19426*

Caenorhabditis elegans unc-13 mutants express decreased neuronal activity and thus are a good model strain for examining defective nervous systems. These *unc-13* mutants as well as wild type N2 strains, show rapid mortality when under oxidative stress. However, the antioxidant vitamin E may prolong survival in *unc-13* mutant and N2 strains under oxidative stress. The addition of vitamin E to organisms under oxidative stress has a protective effect in both N2 and *unc-13 C. elegans* strains. Interestingly, vitamin E resulted in a greater increase in survival rate in N2 worms than with *unc-13* mutant worms. While both strains displayed lower mortality rates with the addition of vitamin E, this finding suggests that vitamin E more efficiently increases survival rates of *C. elegans* with typical nervous system function. The efficacy of vitamin E implies that use of antioxidants may lessen the damage caused by oxidative stress in both N2 and mutant worms.

Abbreviations: ROS- Reactive Oxygen Species

Keywords: antioxidant, neurodegeneration, reactive oxygen species, survival rate, vitamin E,

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Introduction

Oxidative Stress and Reactive Oxygen Species Production

Reactive oxygen species (ROS), such as superoxide anion ($\bullet O_2$-), hydroxy radical (•OH), and hydrogen peroxide (H_2O_2), are compounds that are chemically reactive. This high reactivity may be due to the presence of unpaired valence shell electrons that are referred to as free-radicals. ROS are formed as byproducts of normal metabolic processes that use molecular oxygen and play important roles in many cellular processes, including cell proliferation and signaling, apoptosis, and homeostasis (Coyle and Puttfarcken, 1993; Ames et al., 1993; Afanas'ev, 2005). When subjected to environmental stressors like high temperature or toxic substances, levels of ROS can greatly increase, leading to damage to cellular components and cell apoptosis (Ames et al., 1993). This is known as oxidative stress.

An overabundance in ROS, like that seen during oxidative stress, can lead to cellular damage to lipids, proteins, and nucleic acids (Ames et al., 1993; Sies, 1997). This damage can lead to DNA mutations, misfolded proteins, altered protein interaction, or altered membrane functioning, all of which can lead to cell death and neurodegeneration. In fact, one of the most popular theories of the molecular basis of aging is the oxidative stress hypothesis of aging, also called the free radical hypothesis, and it proposes that age-related physical and mental decline is caused by the gradual accumulation of damage to macromolecules by ROS (Ames et al., 1993). Knowing that the human brain consumes a high level of oxygen, and thus may be especially vulnerable to oxidative stress, has led to many studies of the relationship between ROS and neurodegeneration. Alzheimer's disease (AD) and Parkinson's disease (PD) are both disorders in which there is progressive degeneration seen within certain subsets of

neurons. Current studies have implicated ROS in the mediation of cell death within AD and PD, but the mechanism of the neurodegeneration is still unknown (Clark et al., 2010; Osakada et al., 2003; Busciglio and Yankner, 1995). Further research to clarify the mechanism would allow for more effective treatments to be developed.

Antioxidants

Antioxidants are thought to protect against stress by reducing macromolecular degradation (Martindale and Holbrook, 2002; Benedetti et al., 2008). For example, treating cells with antioxidants prior to stresses such as heat shock, which can result from activation of oxidizing agents, can decrease the damage to the cell (Gorman et al., 1999). This protective effect due to antioxidants is seen in the protection of pancreatic beta-cells against glucose toxicity in diabetic patients (Kaneto et al., 1999). Overexpression of antioxidants may increase longevity, lifespan, and survival due to its ability to protect against oxidative stress (Benedetti et al., 2008).

Vitamin E has been shown to have an antioxidant effect by scavenging ROS and inhibiting ROS formation (Epstein and Gershon, 1972; Clark et al., 2010). Multiple studies have shown that vitamin E has mitigated the negative effects of oxidative stress in various models, both animal and human (Brown et al., 1994; Harrington and Harley, 1988; Hamad et al., 2010).

When comparing antioxidant treatment efficacy in the *C. elegans* model, using vitamin E alone provides the greatest protection in terms of aging when compared to combinations of antioxidants such as vitamin C and vitamin E (Harrington and Harley, 1988). However, at high levels, vitamin E can have toxic effects, especially on the reproduction (Harrington and Harley, 1988; Benedetti et al., 2008). so it is essential to find the concentration that can provide the most protection from oxidative stress, while minimizing toxicity to the organism.

Vitamin E and Nervous System Protection

Vitamin E is a lipid-soluble antioxidant that has four tocopherol isomers (α, β, γ, δ) and four tocotrienol isomers (α, β, γ, δ) and performs chain-breaking reactions. Recent studies have shown that the tocopherol isomers and tocotrienol isomers that were once thought to play a role in antioxidant protection have other important protective actions. Antioxidants protect organisms by becoming oxidized themselves, thus reducing oxidation of other molecules, which prevents ROS damage to the cell (Sies, 1997). Tocopherol isomers have roles in the modulation of signal transduction and gene expression in various cell lines, and tocotrienol isomers possess powerful neuroprotective, anti-cancer, and cholesterol lowering properties (Komiyama et al., 1989). This knowledge prompted further study into the neuroprotective properties of vitamin E. One study suggested that α-tocopherol protects striatal neurons via the reduction of oxidative stress by decreasing intercellular $\cdot O_2^-$ radicals and inhibiting apoptosis (Osakada et al., 2003).

In other models vitamin E has also been shown to have a beneficial effects on an organism undergoing oxidative stress (Benedetti et al., 2008; Harrington and Harley, 1988; Clark et al., 2010; Seis , 1997). For example, Hamad et al., 2010 reported that vitamin E, specifically the water-soluble form Trolox, can help protect the yeast *Schizosaccharomyces pombe* from oxidative stress that was induced with H_2O_2. This protective effect is due to its ability to scavenge ROS intracellularly, thus providing a protective effect against oxidative damage induced by oxidative stress in fission yeast.

The Present Study

Wild type (WT, N2 strain) and *unc-13* strains of *Caenorhabditis elegans (C. elegans)* under oxidative stress were utilized as a model to assess nervous system protection with supplemental vitamin E. The *unc-13* mutation affects the nervous system, giving the otherwise active worms a sluggish and uncoordinated phenotype. The mutations result in increased acetylcholine accumulation and deficiency in

release of many neurotransmitters (Miller et al., 1996; Ahmed et al., 1992). The mutations within the nervous system lead to decreased nervous system functioning.

Research investigating how oxidative stress affects the survival of *unc-13* mutant worms when given a supplement of vitamin E is lacking. Therefore, the goal of this study was to determine if treating with vitamin E would decrease the effect of oxidative stress induced from H_2O_2 on *C. elegans* with normal nervous system function, as well as *C. elegans* with decreased nervous system function.

We hypothesized that the induction of oxidative stress would greatly reduce the survival rates of the worms with decreased nervous system function in comparison to the WT worms with normal nervous system function. Further, we hypothesized, that treating with vitamin E would increase the survival rates of both strains of worms (*unc-13* and N2). Our findings suggest that treating with vitamin E was most effective at increasing survival rates in the worms with normal nervous system function.

Materials and Methods

Growth and Maintenance of C. elegans

Two strains of *C. elegans* were used, the WT strain N2, and CD1091 *unc-13 (e1091)*. Mutants CD1091 *unc-13 (e1091)* were obtained from the *C. elegans* Genetic Center (St. Paul, MN). *C. elegans* were grown and maintained at the adult stage of development on nematode growth medium (NGM) and stored at 20^{o}C. *C. elegans* were maintained by transferring 10-20 adults via picking method to new NGM plates every four days to replenish food source. Standard 12mL NGM plates were prepared according to the protocol described by Sulston and Brenner (1974). A total of 747 *C. elegans* were observed within this study.

Vitamin E

Trolox (6-Hydroxy-2,5,7,8- tetramethyl-chroman-2-carboxylic acid), is a water-soluble derivative of vitamin E and is cell permeable (Benedetti et al., 2008). The Trolox form of vitamin E was used because it was the most successful at reducing oxidative damage in other studies as compared to other vitamin E derivatives (Benedetti et al., 2008). The vitamin E (Trolox, Sigma) concentrations used in the present study (1-3mM) were based upon preliminary studies (Benedetti et al., 2008). The concentrations 1mM, 2mM and 3mM were also specifically tested in this study.

Survival Rate Assay

The four specific plates used were control (NGM), oxidative stress (H_2O_2), vitamin E control (NGM with vitamin E), (Aldrich), and oxidative stress and vitamin E (H_2O_2 with vitamin E).

The H_2O_2 plates were prepared using standard 12mL NGM plates with the final H_2O_2 concentration of 0.99 mM. NGM with vitamin E plates had either 1mM, 2mM, 3mM to assess protective abilities at different final concentrations. These plates were made by adding a vitamin E solution to the NGM plates that sat in a 65^{0}C hot water bath for one hour after removal from the autoclave.

Survival was determined by a 12 hour assay that monitored the survival rates of adult WT and adult mutant worms on the four types of plates. Ten adult worms were transferred using picking method from a NGM stock plate to each of the four plates described above (control, oxidative stress, vitamin E only (1mM, 2mM and 3mM) and oxidative stress and vitamin E). Survival was tested at the ime of plating and 4, 8 and 12 hours after. The assay concluded at 12 hours because the majority of the worms were dead at that point. Survival was determined by nose poke test at each hour. A total of 41 trials were conducted on a total of 747 *C. elegans* worms.

Statistical Analysis

Descriptive statistics were performed to determine the observed survival percentages for *C. elegans* under each experimental condition at 0, 4, 8, and 12 hours. The percent survival of worms was calculated using the number of

worms that were still living in the trial divided by the total number of worms used in the trial (Table 1)

The data was then used to fit an appropriate statistical model after careful consideration of the study design. Since the *C. elegans* were observed only at 0, 4, 8, and 12 hours, the exact time of death for an individual *C. elegans* is known only to fall in a 4 hour interval. Further, for *C. elegans* that survived for the duration of the study, the exact time of death is known only to occur after the final 12 hour assay. These two aspects of the data suggest interval and right censoring, respectively. An appropriate statistical model to deal with these types of censored survival times is a complementary log-log model, which is a discrete analog of the continuous proportional hazards model (Prentice and Gloeckler, 1978; Allison, 1982). Therefore, a complementary log-log model for interval-censored survival times was fit to predict the probability of survival and investigate any statistically significant differences in the survival of *C. elegans* under different experimental conditions.

Preliminary determinations of the individual unadjusted relationships between survival of *C. elegans* and oxidative stress, vitamin E, and *unc-13* mutation was done using three separate survival models, each containing a binary indicator for the presence or absence of the main effect of interest. In addition, an adjusted model was fit, which included binary indicators for all three main effects and the interaction between vitamin E and oxidative stress. Model estimates from this adjusted model were then used to construct estimated survival curves corresponding to each of the experimental conditions. For all models, statistical significance of the appropriate chi-square statistics was assessed at the 0.05 significance level. All analysis was done using SAS 9.2.

Results

Descriptive Statistics

. Data from the three concentrations of vitamin E indicate that 1 mM vitamin E solution yielded the highest percent survival in both N2 and *unc*-13 worms (Figure 1). However, in looking at the percent survival amongst all concentrations of vitamin E there seems to be only slight differences. We felt it was compelling to create models that would predict for trends amongst the varied conditions to provide statistical significance and to aid in looking at future results.

Inferential Statistics

Complementary log-log models for interval-censored survival time were fit using all 747 worms regardless of the vitamin E dosage administered. The unadjusted models suggest that oxidative stress, the presence of the unc_13, and vitamin E exposure all had a significant negative impact on survival when considered individually ($p < 0.001$, $p = 0.009$, and $p<0.001$, respectively). However, further investigation based on the adjusted model revealed a statistically significant interaction between vitamin E and oxidative stress after controlling for the presence of the *unc-13* mutation ($p < 0.001$). The results from the three different concentration of vitamin E were combined in order to simplify the study design for inferential statistics. This suggests that the effect of vitamin E on the survival of *C. elegans* differs depending on their exposure to oxidative stress. More specifically, as shown in Figure 2, when controlling for the presence of the *unc-13 mutation*, vitamin E improves the survival of *C. elegans* exposed to oxidative stress ($p = 0.005$), but has a detrimental effect on the survival of *C. elegans* not exposed to oxidative stress ($p < 0.001$). The adjusted model also suggests that the presence of the *unc-13* mutation has a negative impact on survival of *C. elegans* after controlling for oxidative stress and vitamin E exposure ($p < 0.001$).

Table 1: Descriptive Statistics for *C. elegans* by strain, oxidative stress exposure and Vit E exposure (n=747)

Characteristic	0-4 hours	4-8 hours	8-12 hours

WT: Control	100% (n=123)	100% (n=123)	100% (n=123)
WT: Vit E	100% (n=73)	98.6% (n=72)	80.8% (n=59)
WT: Oxidative Stress	39.6% (n=58)	22.9% (n=16)	11.5% (n=11)
WT: Oxidative Stress + Vit E	50.6% (n=39)	27.8% (n=18)	1.26% (n=21)
unc-13: Control	100%(n=121)	100% (n=121)	93% (n=112)
unc-13: Vit E	79.7% (n=12)	67.8% (n=7)	44.1% (n=14)
unc-13: Oxidative Stress	29.2% (n=68)	3.1% (n=25)	0% (n=3)
unc-13: Oxidative Stress + Vit E	44.7% (n=42)	18.4% (n=20)	6.6% (n=9)

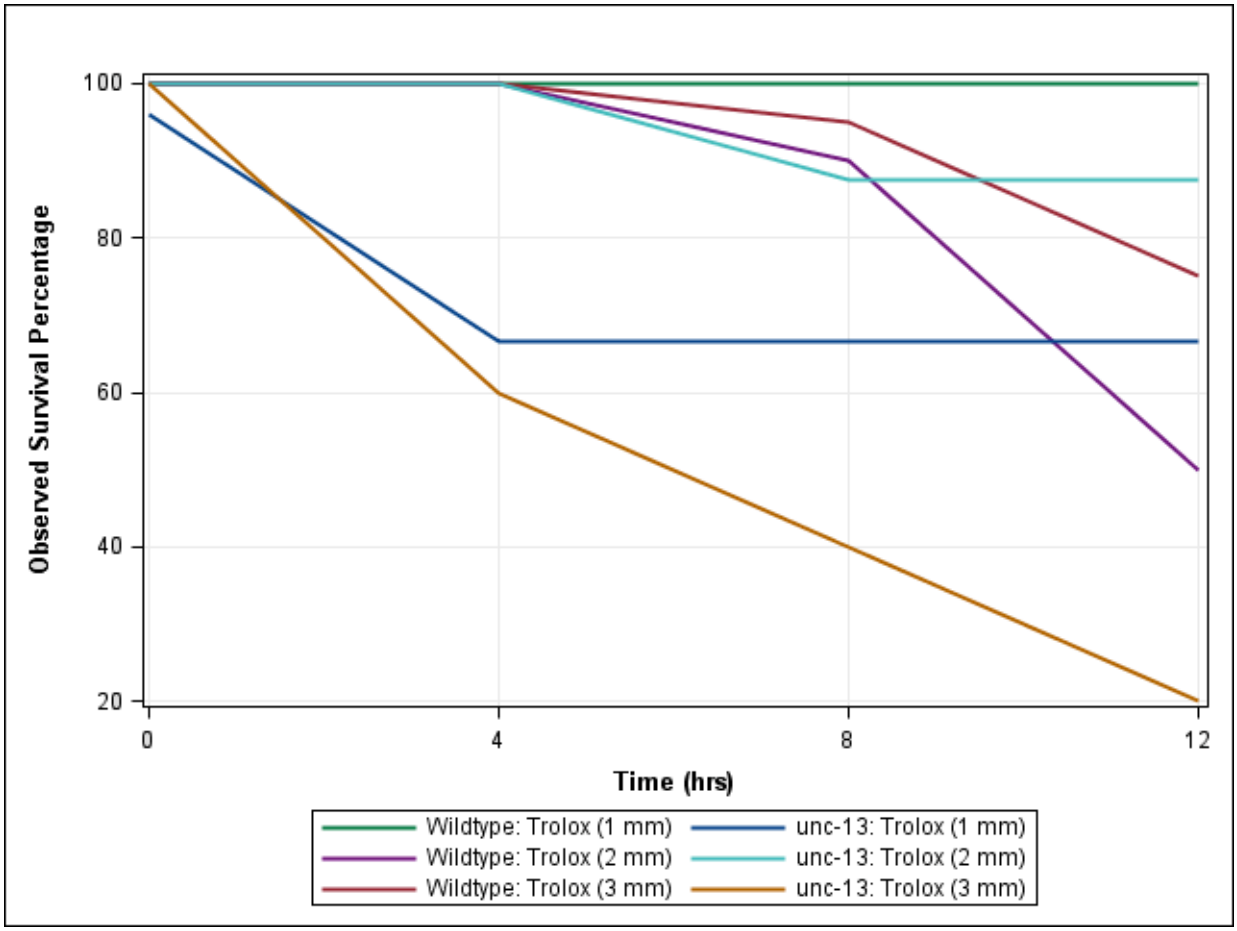

Figure 1: Percent survival of *C. elegans* on agar plates with 1 mM, 2 mM and 3 mM concentrations of vitamin E in both the WT strain and *unc-13* mutant worms over a 12 hour time period. Percent survival was calculated with N=747 worms over six trials per condition.

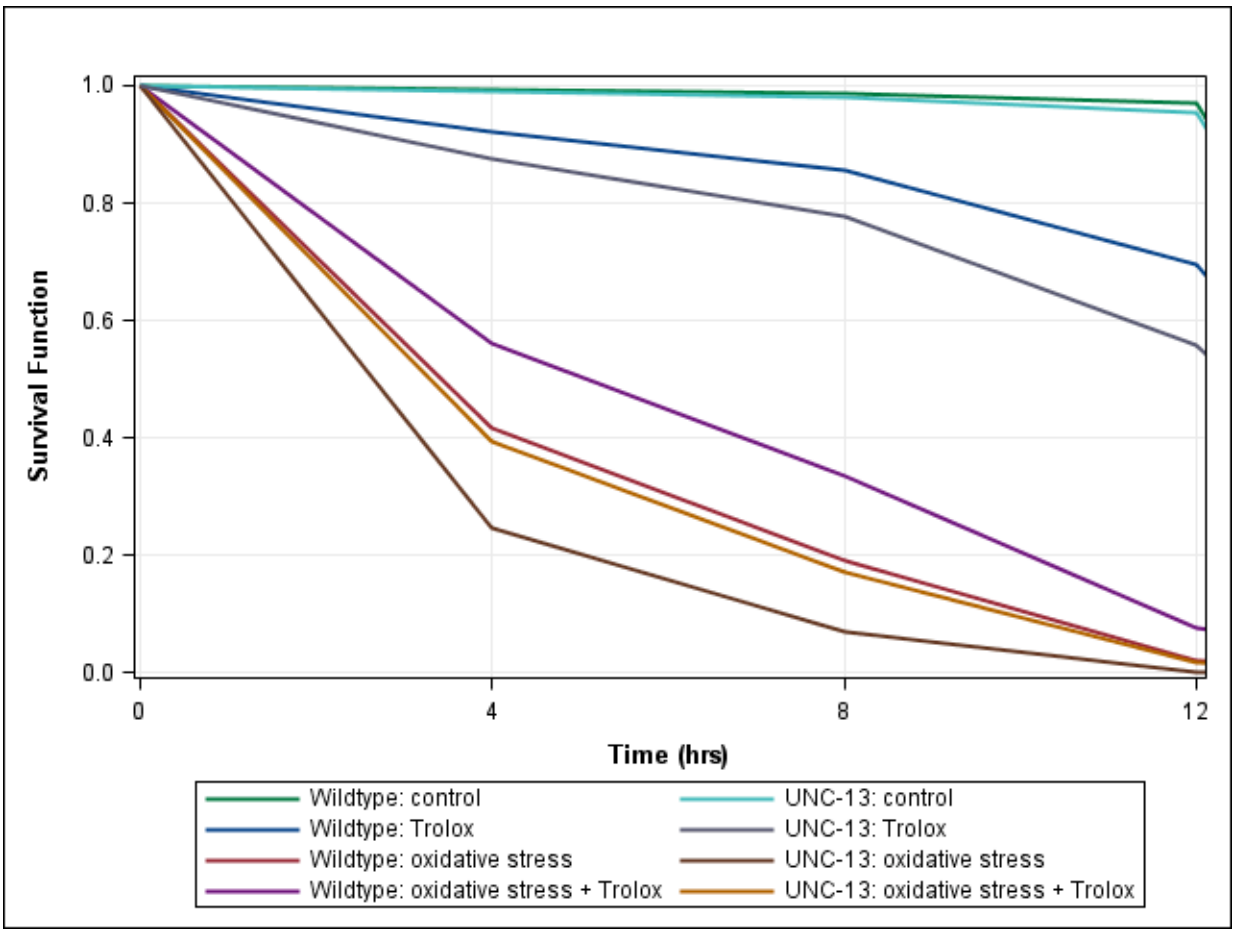

Figure 2: Predicted survival for *C. elegans* by strain, oxidative stress exposure and vitamin E exposure (n=747). vitamin E concentrations (1mM, 2mM and 3mM) were combined within the predictive model.

Discussion

This study examined the effects of vitamin E on survival rates of both mutant and WT worms undergoing oxidative stress. In confirmation of our hypothesis, the results for both strains showed greatly reduced levels of survival function when treated with H_2O_2 as compared to NGM-only controls. Additionally, *unc-13* mutants showed lower levels of survival function than WT worms when subjected to oxidative stress, possibly because these specific mutant worms have decreased nervous system activity, which makes them more susceptible to the damages of oxidative stress.

Further, we also observed through our results that vitamin E was detrimental to survival function when comparing both strains of worms plated on NGM only. These results may be observed because vitamin E at higher concentrations may have a toxic effect on worms, which is consistent with the findings of Benedetti et al., 2008, though our concentrations were slightly lower. Our results indicated that vitamin E did have a significant effect on the survival function of both WT and *unc-13* worms when under increased oxidative stress due to H_2O_2 (Fig. 1). This suggests that vitamin E can help combat the negative effects of oxidative stress as seen by the increase in survival function.

Effect of Different Concentrations of vitamin E on C. elegans *Strains*

The effects of vitamin E differed slightly with varying conditions. However, survival rates of *C. elegans* remained high for nearly all vitamin E concentrations within the range 1 mM to 3 mM. Only after 12 hours at 3mM was it observed that the *unc-13* mutants had only 20% survival., while the WT worms retained almost 90% survival. This finding could suggest that for worms with decreased neuronal function, higher concentrations of vitamin E may have a detrimental effect on survival and that vitamin E is toxic to these mutated worms at lower levels than it is for WT worms. However, the lower concentrations provided a slightly protective effect against oxidative stress even for the mutants.

Protective Effect of vitamin E on Neurodegeneration

It is interesting to note that within our model, the survival function of *unc-13* worms treated with vitamin E and H_2O_2, as seen in Figure 1, closely resembles that of WT worms treated with H_2O_2. Treating the *unc-13* mutants with vitamin E may boost their ability to cope with oxidative stress almost to the level of WT worms. If the nervous system of *unc-13* worms contained a defect in ROS metabolism similar to that seen in human nervous system disorders, a parallel could be drawn to explain the beneficial effect that vitamin E had on *unc-13* worms. As an ROS scavenger, vitamin E may counteract the defect in ROS metabolism, bringing the level of ROS metabolism back to normal levels for the *unc-13* mutant worms.

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Corresponding Author

Jessica Porcelan
Ursinus College
jeporcelan@ursinus.edu
6152 Ludgate Circle
Mechanicsburg, PA 17050

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