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Chronic vitamin E deficiency promotes vitamin C deficiency in zebrafish leading to degenerative myopathy and impaired swimming behavior

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Abstract

We hypothesized that zebrafish (*Danio rerio*) undergoing long-term vitamin E deficiency with marginal vitamin C status would develop myopathy resulting in impaired swimming. Zebrafish were fed for 1 y a defined diet without (E–) and with (E+) vitamin E (500 mg α -tocopherol/kg diet). For the last 150 days, dietary ascorbic acid concentrations were decreased from 3500 to 50 mg/kg diet and the fish sampled periodically to assess ascorbic acid concentrations. The ascorbic acid depletion curves were faster in the E– compared with E+ fish ($P < 0.0001$); the estimated half-life of depletion in the E– fish was 34 days, while in it was 55 days in the E+ fish. To assess swimming behavior, zebrafish were monitored individually following a “startle-response” stimulus, using computer and video technology. Muscle histopathology was assessed using hematoxylin and eosin staining on paramedian sections of fixed zebrafish. At study end, E– fish contained 300-fold less α -tocopherol ($p < 0.0001$), half the ascorbic acid ($p = 0.0001$) and 3-fold more malondialdehyde ($p = 0.0005$) than did E+ fish. During the first minute following a tap stimulus ($p < 0.05$), E+ fish swam twice as far as did E– fish. In the E– fish, the sluggish behavior was associated with a multifocal, polyphasic, degenerative myopathy of the skeletal muscle. The myopathy severity ranged from scattered acute necrosis to widespread fibrosis and was accompanied by increased anti-hydroxynonenal staining. Thus, vitamin E deficiency in zebrafish causes increased oxidative stress and a secondary depletion of ascorbic acid, resulting in severe damage to muscle tissue and impaired muscle function.

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Keywords

alpha-tocopherol; ascorbic acid; ataxia; defined diet; myopathy; neuropathy; vitamin E deficiency symptoms

1. Introduction

Vitamin E is a potent, lipid-soluble antioxidant that scavenges peroxy radicals, preventing radical-mediated autooxidation of lipids (Atkinson et al., 2010; Traber and Atkinson, 2007). Ataxia and associated myopathic lesions caused by α -tocopherol deficiency has been reported in humans (Binder et al., 1967; Guggenheim et al., 1982; Higuchi et al., 1993; Kobayashi et al., 1984; Neville et al., 1983) and other primate species (Hayes, 1974; Juan-Salles et al., 2003; Nelson et al., 1981), rodents (*rodentia*) (Thomas et al., 1993), and guinea pigs (*Cavia porcellus*) (Hill et al., 2001). Histopathologically, vitamin E deficiency causes muscle fibers to vary in size and there is proliferation of sarcolemmal nuclei, vacuolization, mononuclear infiltration, and scattered necrosis. The neurological and myopathologic symptoms of vitamin E deficiency in humans can be ameliorated, and in some cases reversed by supplementation with supranutritional doses of vitamin E (Traber, 2006). Moreover, reversal of myopathy with dietary vitamin E has been used as a basis for evaluating its biopotency in experimental vitamin E deficiency in rats (*ratus*) (Machlin et al., 1982). Although the clinical symptoms, cause, and treatment of vitamin E deficiency are well documented, the mechanisms by which vitamin E functions within the musculature and nervous systems, and how its deficiency results in neuromyopathies, remain unknown, but some progress has been made. In vitro, α -tocopherol promotes membrane repair in response to oxidant challenge, but does not prevent the initial damage (Howard et al., 2011). Additionally, in vitamin E deficient α -tocopherol transfer protein knockout mice (*Mus musculus*), various genes were over-expressed (> 10-fold) in ataxic muscles (Vasu et al., 2009).

The evidence in humans that α -tocopherol functions as a lipid soluble antioxidant is limited, but because cigarette smoking generates free radicals (Pryor, 1992), α -tocopherol plasma depletion kinetics were compared in smokers and non-smokers. Smokers had greater lipid peroxidation (F₂-isoprostane concentrations) and faster plasma α -tocopherol disappearance rates (Bruno et al., 2005). When smokers were supplemented with vitamin C (500 mg twice daily for 2 weeks), α -tocopherol disappearance rates were normalized to rates observed in non-smokers (Bruno et al., 2006). These findings are consistent with the role of ascorbic acid recycling the α -tocopheroxyl radical, which has been shown in vitro (Buettner, 1993). In vivo, vitamin E status may critically depend upon the adequacy of vitamin C status and models to assess α -tocopherol function need to take into account whether the animal model can synthesize ascorbic acid.

Zebrafish (*Danio rerio*) are a robust model to study the pathogenesis of muscle dystrophies (Guyon et al., 2007; Steffen et al., 2007) and neuropathies (Bandmann and Burton, 2010; Ingham, 2009; Xi et al., 2011). Additionally, zebrafish exhibit complex behaviors that are increasingly used to model neurobehavioral disorders (Cachat et al., 2011; Egan et al., 2009), to probe neuromuscular circuit activity (Issa et al., 2011), and assess long-term consequences of toxic chemical exposures (Truong et al., 2012). Previously, we reported that vitamin E deficient adult zebrafish have reduced swimming behaviors compared with vitamin E sufficient zebrafish, suggestive of either a neuropathy, myopathy, or combination of the two disorders, but the underlying pathology was not determined (Miller et al., 2012).

Zebrafish, like humans (Chakraborti and Bahnson, 2010), express gluconolactonase (GNL) [EC3.1.1.17], a lactone-hydrolyzing enzyme (Fujisawa et al., 2011), which is the penultimate enzyme in the pathway for ascorbic acid synthesis and whose knockdown in mice causes vitamin C deficiency (Kondo et al., 2006). Humans require vitamin C because they lack a functional gulonolactone oxidase (GLO) (Nishikimi and Yagi, 1991). Zebrafish, a member of the teleost fish, likely lacks GLO because teleost fish lack GLO (Dabrowski, 1990; Touhata et al., 1995; Toyohara et al., 1996), overexpression of GLO in medaka fish reverses vitamin C deficiency and addition of ascorbic acid to the diet protects zebrafish, especially males, from scurvy (Dabrowski and Ciereszko, 1996; Phromkunthong et al., 1994). Drouin et al (Drouin et al., 2011) suggest that BLAST searches fail to identify the GLO gene in zebrafish because teleost fish have a complete loss of the GLO gene.

We hypothesized that vitamin E deficiency results in a degenerative myopathy in zebrafish, similar to other model vitamin E deficient organisms. We also propose that zebrafish because they, like humans, require dietary vitamin C are a useful model for investigating the pathological consequences of vitamin E deficiency combined with vitamin C insufficiency. Therefore, zebrafish were raised on either a vitamin E deficient or sufficient diet, and then the fish were transferred to diets that had minimal levels of dietary vitamin C to assess whether vitamin E deficiency depletes ascorbic acid concentrations more rapidly, and causes pathological changes in histologic measures and in swimming behaviors.

2. Methods

2.1 Fish husbandry

Tropical 5D strain zebrafish (*Danio rerio*) were housed in the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University and studied in accordance with protocols approved by the Institutional Animal Care and Use Committee. Adult zebrafish were housed under standard laboratory conditions, as previously described (Lebold et al., 2011; Miller et al., 2012). Beginning at 4 w of age, zebrafish were fed a defined experimental diet as previously described (Lebold et al., 2011; Miller et al., 2012). Defined diets were prepared in 300 g batches without (E⁻) or with vitamin E (E⁺, 500 mg *RRR*- α -tocopherol/kg diet) and stored at -20 °C until fed to the zebrafish. These defined diets contained 3500 mg ascorbic acid/kg diet, which was added as StayC (10 g/kg diet, Argent Chemical Laboratories Inc, Redmond, WA, USA).

To assess whether minimal vitamin C intakes potentiated vitamin E deficiency symptoms, the vitamin C was decreased to low, but adequate levels. As noted by Fournier et al. (2000), information concerning ascorbic acid requirements for fish are scarce. We estimated the requirements at a range of 10–20 mg ascorbic acid/kg diet for zebrafish; to be in somewhat excess, we therefore added 50 ascorbic acid mg/kg diet, based on estimates of vitamin C needed by freshwater, fin fishes by the Committee on Animal Nutrition Board, Agriculture National Research Council (Committee on Animal Nutrition Board on Agriculture National Research Council, 1993). At the time of the studies reported herein, the E⁻ and E⁺ diets each contained ascorbic acid at 50 mg/kg diet. It should be noted that this level of ascorbic acid/kg was found to be inadequate for rapidly growing zebrafish (Kirkwood et al., 2012). The dietary vitamin E contents were measured (E⁻ diets: α -tocopherol 1.4 ± 0.1 , γ -tocopherol 1.9 ± 0.1 mg/kg diet; E⁺ diets: α -tocopherol 300 ± 22 , γ -tocopherol 4.5 ± 0.4 mg/kg diet).

Initially, zebrafish were fed from age day 30 for the next 200 days on the defined diets containing 3500 mg ascorbic acid/kg diet to allow adequate growth, they were then fed for the next approximately 145 days on the defined diets containing 50 mg ascorbic acid/kg diet to reduce the amount of ascorbic acid available for vitamin E recycling, and thus make

vitamin E deficiency more severe. To assess ascorbic acid depletion, fish from each diet group (n=3/group) were analyzed with respect to body weight and ascorbic acid concentrations at days 0, 18, 34, 52; these fish were not fasted. Following the behavior studies (described below) at 139 (n=5 per diet group) and 144 days (E- n=6, E+ n=5), fish were also harvested for ascorbic acid and malondialdehyde (MDA) analyses; these fish were fasted approximately 24 h. Body weights from all fish (E- n=32, E+ n=30, lab n=30) that were harvested at the end of the study were averaged and shown as day 142 on diet in Figure 1.

2.2 Behavior testing

To evaluate swimming behavior, computer-assisted video monitoring and tracking software was used based on a modified method of Miller et al (Miller et al., 2012). After approximately 145 days on the low ascorbic acid diet, E+ and E- zebrafish (E- n=31, E+ n=30) were placed in 1.75 L tanks (one fish per tank), given 24 h to acclimatize prior to beginning the trials and fasted for the duration of the behavior trials. Tanks were set on shelves with the broadside facing the camera, separated by dividers, evenly backlit, and kept on a 14 h light/10 h dark photoperiod. Water temperature was maintained at 28 °C.

Automated electro-magnetic solenoids were used to generate a tap on the base of the tanks to elicit a “startle-response”. Trials were recorded using a Sony HD camcorder (Sony Handycam HDR-SR11) coupled with the Noldus Etho-Vision XT V 7.0 analysis software (Leesburg, VA, USA). Three trials of 6 min without a stimulus were used for baseline swimming behavior and results from each fish were averaged; individual outcomes from each fish are reported as average distance swam per minute. Two trials were then conducted; the first trial used single tap stimulation, the second trial used multiple taps. Following the single tap, swimming speeds and distances were recorded; results from each fish are reported as distance swam per minute for each minute of the 6 min immediately following the tap. For the multiple tap trial, the solenoid struck the tanks once every 5 s for 90 s (18 taps total). Following the first tap, swimming speeds and distances were recorded; results from each fish are reported as distance swam per minute for each minute of the 6 minutes immediately following the first tap. Fish were allowed to rest for one hour between the single and multiple tap trials.

2.3 Measurement of α -tocopherol

One h after the behavior trials were completed, fish were euthanized by an overdose of tricaine (Sigma-Aldrich, St Louis, MO, USA), flash frozen in liquid nitrogen, and stored at -80 °C until analyzed. Whole fish α -tocopherol concentrations (E-, E+, and lab n=10/group) were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ECD), as previously described (Lebold et al., 2011; Podda et al., 1996). Briefly, whole fish were saponified in the presence of alcoholic KOH with 1% ascorbic acid, cooled, extracted with hexane, then the extract was dried under nitrogen and the residue resuspended in methanol for injection into the HPLC system. α -Tocopherol concentrations were determined by comparison to standard curves generated from authentic compounds.

2.4 Measurement of malondialdehyde (MDA) and ascorbic acid

Following the switch to low vitamin C diets, at indicated intervals, zebrafish were collected for ascorbic acid analyses (n=3/group). In addition, one h after the behavior trials were completed, fish (E-, n=11, and E+ or lab, n=10/group) were euthanized by an overdose of tricaine, weighed, and homogenized in buffer (5% trichloroacetic acid [TCA, Sigma-Aldrich], 0.08% diethylenetriaminepenta-acetic acid [DTPA, Acros Organics, Morris Plains, NJ, USA], 250 mM perchloric acid [PCA, Fisher Scientific, Fair Lawn, NJ, USA], and 0.4 mM dithioerythritol [DTE, Sigma-Aldrich]). Homogenates were then centrifuged, the

supernatants collected, divided into two aliquots (one for MDA analysis, one for ascorbic acid analysis), flash frozen in liquid nitrogen, and stored at -80°C .

Ascorbic acid concentrations were determined by HPLC-ECD, as described (Frei et al., 1989). MDA concentrations were determined using a modified method of Hong et al (Hong et al., 2000). Briefly, the supernatants (200 μL) were mixed with 0.2% BHT (20 μL) and 10 N NaOH (100 μL), incubated for 30 min at 60°C , cooled to room temperature, mixed with 10% H_3PO_4 (1 mL) and cooled on ice for 5 min. An aliquot (250 μL) was then transferred to a fresh tube, mixed with 98% thiobarbituric acid (TBA, 150 μL), incubated for 30 min at 95°C , cooled on ice, mixed with butanol (300 μL), and centrifuged at 16,000 g (Eppendorf, Hauppauge, NY, USA) at 10°C for 5 min. The supernatant was then transferred to injection vials. 1,1,3,3-tetraethoxypropane standards were prepared as previously described (Young and Trimble, 1991). MDA separation was performed using a Shimadzu HPLC (Kyoto, Japan) with a Phenomenex Luna C18 column (250×4.6 mm, 5 μm particle size, Torrance, CA, USA) and a mobile phase consisting of 50% (v/v) methanol and 50% phosphate buffer (25 mM KH_2PO_4). Detection of MDA-TBA adducts was performed using a Shimadzu RF-10A spectrofluorometric detector with a xenon short arc lamp (Ushio Inc, Tokyo, Japan) at the following wavelengths: excitation 532 nm, emission 553 nm. Zebrafish MDA concentrations were calculated by comparisons to the standard curve, and then expressed per g body mass.

2.5 Histopathology

One h after the behavior trials were completed, fish ($n=10/\text{diet group}$) were euthanized by an overdose of tricaine, and fixed in 10% buffered formalin (Azer Scientific, Morgantown, PA, USA) for at least three days. Fish were then transferred to Cal-Ex® Decalcification solution (Thermo Fischer Scientific, Hampton, NH, USA) for two days, removed and rinsed with water, and placed back into 10% buffered formalin. Fish were submitted to the Veterinary Diagnostic Laboratory at Oregon State University and processed for routine histology. Paramedian sections were prepared from each fish and embedded in paraffin. Three-micrometer sections were stained with hematoxylin and eosin (H&E) according to the manufacturer's instructions using the GLX Slide Stainer (Baxter, McGaw Park) or used for Immunofluorescence studies as described below; recuts of some fish were stained with Trichrome. A board-certified veterinary pathologist (CVL) performed histopathological analysis by light microscopy.

2.6 Immunofluorescence

An antibody to hydroxynonenal (HNE, ab48506, Abcam, Cambridge, MA, USA) was used for immunofluorescence (IF) to analyze muscle tissue in paraffin-embedded sections from five fish per group (E- and E+), as well as one negative control fish. Slides were prepared according to the BioLegend protocol (steps 9–18 of IF protocol paraffin-embedded sections; San Diego, CA, USA) with the exception that the samples were incubated with the primary antibody (1:250 in 0.5% BSA) overnight at 4°C . Following step 18 of the BioLegend protocol, slides were rinsed twice for 7 minutes each in phosphate buffered saline (PBS), followed by the addition of the secondary antibody (Alexa fluor 555 goat anti-mouse 1:1000 in 0.5% FBS) for 2 h. Slides were then rinsed three times for 10 minutes each in PBS. Slides were then mounted with a coverslip containing Invitrogen Prolong Gold anti-fade reagent. The primary antibody was excluded for the negative control. Slides were viewed/photographed under a Rhodamine filter. Fluorescence intensity was quantified using ImageProPlus (Media Cybernetics, Bethesda, MD, USA).

2.7 Statistics

Statistical analysis was performed using Prism 6.0b for Mac OS X (GraphPad Software, Inc., La Jolla, CA, USA). When unequal variances were observed between groups, the data was logarithmically transformed, and then statistics were performed on the normalized data. Comparisons between E⁻ and E⁺ groups' antioxidant, MDA concentrations, and HNE fluorescence intensity were evaluated using an unpaired t-test. Comparisons between E⁻ and E⁺ groups in response to stimulus and comparisons between single and multiple tap stimulus were evaluated using a repeated measures two-way ANOVA; paired comparisons were used for post-hoc analysis (Tukey's or Bonferroni multiple comparisons, as recommended by the software). Differences were considered significant at $p < 0.05$. Values shown are expressed as mean $\pm$ SD. SEM are shown in Figure 2.

Ascorbic acid depletion rates were calculated using logarithmic-transformed individual data at each time point; slopes were compared using an extra-sum of squares, F-test (GraphPad Prism). The half-lives were estimated from the equation: half life = $\log(10)/\text{slope}$ of the line estimated from the log (ascorbic acid concentrations) over time.

3. Results

3.1. Whole body weights, ascorbic acid, α -tocopherol, and MDA concentrations

At age approximately 6 months following 200 days on the defined diets, the E⁻ and E⁺ zebrafish were switched to the low ascorbic acid defined diets and were followed for the next approximately 145 days (Figure 1). At the time the fish were switched to the low ascorbic acid diets, there were no significant differences in body weight between the groups (E⁻, E⁺ or lab); by 52 days on diet, the body weights of the lab diet fish were greater ($P < 0.05$) than the defined diet fish; by the end of the study the body weights in all groups were different from each other (Figure 1A, 2-way ANOVA: time $p = 0.0276$, diet $p < 0.00001$, interaction, $P = 0.756$; $P < 0.05$, Tukey multiple comparisons). At the end of the study, lab and E⁺ zebrafish contained similar α -tocopherol concentrations, which were 300 times greater than those of the E⁻ zebrafish (Figure 1B, ANOVA interaction, $p < 0.0001$, $p < 0.05$ Tukey multiple comparisons).

The time course of ascorbic acid depletion was followed over the course of the study (Figure 1C). A linear fit of the logarithmically transformed data showed that the ascorbic acid depletion curves for E⁻ and E⁺ zebrafish were different from each other ($P < 0.0001$), while the lab fish ascorbic acid concentrations did not change significantly. The E⁻ fish were depleted of ascorbic acid with an estimated half-life of 34 days, while in the E⁺ fish the half-life was 55 days. At the end of the study, the E⁺ zebrafish contained three times as much ascorbic acid as the E⁻ fish (Figure 1C, ANOVA $p < 0.0001$, $p < 0.05$, Tukey multiple comparisons). Given that the E⁺ and E⁻ diets contained equal, but low amounts of ascorbic acid, these findings suggest that the α -tocopherol deficiency led to a secondary depletion of ascorbic acid in E⁻ zebrafish. Simultaneously, the E⁻ zebrafish experienced increased lipid peroxidation as evidenced by 3-fold higher MDA concentrations, while MDA concentrations were not significantly different between E⁺ and lab zebrafish (Figure 1D, ANOVA $p < 0.0001$, $p < 0.05$ Tukey multiple comparisons).

3.2. Behavioral testing

To evaluate the severity of the neuromuscular deficits caused by vitamin E deficiency, zebrafish were challenged by a tap stimulus and then their swimming behavior was evaluated. Three comparisons were made: the E⁻ fish were compared with the E⁺ fish following stimuli of a single tap (Figure 2A) or multiple taps (Figure 2B); the distance swam following the two different stimuli by the E⁻, or by the E⁺ fish, at various times were also

compared (Figure 2A vs 2B). Within the first minute following the single tap, the E+ fish swam further than the E- fish tap (Figure 2A). Specifically, the E- fish increased their swimming distance by approximately 40 cm, while the E+ fish increased their swimming distance by approximately 180 cm—more than doubling their baseline swimming distance (two-factor ANOVA, interaction $P=0.0028$; $P<0.05$, Bonferroni post-hoc comparison); by 2 minutes, distances swam by both groups were no longer statistically significant. Within the first minute following the multiple taps, the E+ fish swam further than the E- fish tap (Figure 2B). Specifically, the E- fish increased their swimming distance by approximately 125 cm, while the E+ fish increased their swimming distance by approximately 290 cm—more than tripling their baseline swimming distance (two-factor ANOVA, interaction $P=0.0005$; $P<0.05$, Bonferroni post-hoc comparison); by 2 minutes, distances swam by both groups were no longer statistically significant.

The multiple tap stimulus compared with the single tap (comparing figure 2B with 2A) caused the E- fish to swim approximately 85 cm farther at 1 minute and approximately 75 cm farther at 2 minutes (two-factor ANOVA, interaction $P<0.0001$; $P<0.05$, Bonferroni post-hoc comparison). The multiple tap stimulus compared with the single tap caused the E+ fish to swim approximately 110 cm farther at 1 minute and approximately 145 cm farther at 2 minutes (two-factor ANOVA, interaction $P=0.0002$; $P<0.05$, Bonferroni post-hoc comparison).

3.3. Histopathology

Histopathology was performed to determine the underlying cause of the impaired swimming in E- fish (Figures 3A and B) compared with E+ fish (Figure 3C). E- fish stained with H & E and examined by light microscopy showed that 8 out of 10 fish displayed a multifocal, polyphasic, degenerative myopathy. This defect was characterized by a combination of acute, subacute, as well as chronic, random myofiber degeneration, ranging from a loss of striation and fragmentation of the sarcoplasm to complete loss of the sarcoplasm with collapse of myofibers. These changes were accompanied by mild to moderate interstitial edema and loose interstitial infiltrates of mononuclear inflammatory cells (minimal interstitial myositis). Scattered areas of muscle regeneration were observed that displayed activation of Satellite cells along intact basal laminae (not shown) and formation of myotubes with increased basophilia of sarcoplasm, lack of striation and rowing of large euchromatic nuclei (activated myoblasts). Trichrome stain did not reveal increased collagen deposits (fibrosis) in any of the examined cases. In 4 fish, degenerative myopathy was moderate to severe and widespread involving 50% to 90% of the examined skeletal musculature, while in another 4 cases degeneration was mild. Lesions were particularly severe along the dorsum (around vertebral column) and decreased in severity from cranial (behind the head) to caudal (tail) skeletal muscle.

For the E+ fish examined for histology, 8 out of 10 showed no myofiber damage, while in 2 out of 10, groups of myofibers along the vertebral column or the ventrum showed contraction bands consistent with mild, very acute, monophasic myofiber damage. These subtle changes were interpreted as a consequence of handling during behavioral testing or at euthanasia.

3.4. Localization of 4-hydroxynonenal adducts

Immunofluorescence (IF) employing an antibody to 4-hydroxynonenal (and a fluorescent secondary antibody) was used to assess muscle tissue damage in E- and E+ fish. An antibody to HNE was used to assess oxidative damage in muscle sections because HNE-protein adducts are well known examples of damage caused by reactive aldehydes generated during lipid peroxidation in vivo (Zainal et al., 2000). We found both diffuse and punctate

staining of E⁻ zebrafish muscles with most of the staining along the outside of fibers, i.e. near the sarcolemma (Figure 4A), while the E⁺ fish and the E⁻ negative control were minimally stained (Figures 4B and C, respectively). E⁻ compared with E⁺ fish showed a greater than 3-fold increase ($p < 0.05$) in fluorescence due to antibody staining (Figure 4D).

4. Discussion

Herein we show that chronic vitamin E deficiency, along with marginal vitamin C status in zebrafish results in an increase in oxidative stress biomarkers, a secondary depletion of ascorbic acid, and a degenerative myopathy, accompanied by a marked increase in HNE-adduct formation in muscles. The degenerative myopathy caused significant functional impairments, as illustrated by the impaired ability of the E⁻ zebrafish to respond to a single tap, startle stimulus. These findings are consistent with the known observation that an antioxidant, ethoxyquin, prevents vitamin E deficiency myopathy in rats, but that rapid onset of muscle damage occurred when the 0.1 % ethoxyquin was removed from the vitamin E deficient diet (Gabriel et al., 1980). It is likely that the myopathy of vitamin E deficiency results from a chronic lack of sufficient antioxidant protection, leading to increased lipid peroxidation and that α -tocopherol has a critical role of in preventing muscle damage caused by lipid peroxidation. Notably, the muscle degeneration in zebrafish resembles the myopathies described in vitamin E deficient mammals (Gabriel et al., 1980; Juan-Salles et al., 2003; Machlin et al., 1982; Nelson, 1983; Nelson et al., 1981; Pillai et al., 1994; Thomas et al., 1993; Vasu et al., 2009), confirming that zebrafish serve as a useful model to investigate the mechanisms underlying degenerative myopathy caused by vitamin E deficiency. Indeed, the zebrafish has been touted as an ideal model system for studying myopathies (Guyon et al., 2007).

In this study, E⁻ and E⁺ fish were fed diets containing equal, but low amounts of vitamin C. Notably, vitamin E deficiency caused a secondary ascorbic acid depletion, clearly demonstrating the relationship between these antioxidant vitamins and illustrating important nutrient-nutrient interactions that occur in response to increased oxidative stress. The addition of vitamin C to sea bass (*Dicentrarchus labrax*) diets containing 5% docosahexaenoic acid were found to salvage vitamin E (Betancor et al., 2012), again showing the interactions between these two antioxidant vitamins. In humans, the rate of disappearance of vitamin E in response to oxidative stress is dependent on vitamin C status (Bruno et al., 2005). In guinea pigs, vitamin C deficiency alone does not cause significant muscle damage, but does exacerbate vitamin E or selenium deficiencies and the resultant myopathies (Hill et al., 2003; Hill et al., 2009). Like humans and guinea pigs, zebrafish require both vitamins in the diet. Other model organisms synthesize vitamin C *de novo*, limiting the utility of those models to mimic human vitamin E deficiency myopathies since adequate vitamin C may promote vitamin E recycling and thus prolong the time before vitamin E deficiency symptoms occur. Thus, zebrafish are a particularly applicable organism for investigating vitamin E deficiency. The random, degenerative myopathy largely explains the behavioral abnormalities observed in vitamin E deficient zebrafish; however, we did not examine the peripheral nervous system in this study. The vitamin E deficient zebrafish responded differently to a single tap compared with a multiple tap, raising the question whether the multiple taps overcame a neurologic deficit and elicited a response or caused the fish sufficient agitation that they moved despite their myopathy. The role of muscle innervation in muscle degeneration has been examined previously in rats, where it was found that both the muscle and the nervous system damage occurred independently (Pillai et al., 1994). Indeed, myopathy has been suggested to be a consequence of decreased plasma α -tocopherol concentrations in humans secondary to statin drug use. Statins both lower cholesterol levels and interfere with ubiquinol synthesis, and thus decrease antioxidant protection by both α -tocopherol and ubiquinol in these patients (Galli and Iuliano, 2010).

What is the mechanism by which increased myopathy occurs in the E⁻ zebrafish? Howard et al. (Howard et al., 2011) suggest that vitamin E is necessary to promote membrane repair. They find that vitamin E does not decrease membrane damage in response to an oxidant, but that its absence decreases the effectiveness of repair mechanisms. Given that there are numerous genes and signaling pathways involved in damage repair (Tal et al., 2010), and that muscular dystrophy is defined as “degenerates faster than it can be repaired” (Guyon et al., 2007), vitamin E appears critical for the remodeling of muscle during repair. There are numerous possibilities for the mechanism of vitamin E function in muscle, in addition to its obvious role as a lipid peroxyl radical scavenger (Traber and Atkinson, 2007). The presence of α -tocopherol in the membrane may increase membrane fluidity (Tai et al., 2010) and allow membrane signaling to proceed at a more rapid pace. Atkinson et al (Atkinson et al., 2010) propose that α -tocopherol partitions into non-raft portions of the membrane, where it could more readily protect polyunsaturated fatty acids. In studies of the muscle from vitamin E deficient rats, more than 50 genes were up-regulated (Nier et al., 2006), suggesting that repair mechanisms are up-regulated in vitamin E deficiency. The histologic examination of the E⁻ fish support the idea that multiple regeneration attempts took place with cycles of damage and repair. The accumulation of HNE-adducts in the E⁻ fish also supports the concept that cumulative membrane damage occurred that ultimately destroyed the myofibres. Thus, the increased lipid peroxidation that occurs with vitamin E deficiency more extensively damage the membranes than ultimately can be repaired, leading to over 90% of the skeletal muscle affected by progressive degenerative myopathy (Figures 3).

In conclusion, the vitamin E⁻ deficient zebrafish is an excellent model to study the mechanisms by which insufficient α -tocopherol causes muscle degeneration. The ability to decrease the zebrafish's ascorbic acid protection is especially valuable in this regard. Vitamin E's role in membrane and muscle repair suggests that inadequate α -tocopherol or ascorbic acid status or their combination leads to progressive muscle damage. Thus, the zebrafish are a useful model for mechanistic studies of vitamin E and/or C deficiency.

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Figure 1. Body weights, antioxidant and MDA concentrations in adult zebrafish fed defined diets for 1 y

A. Time 0 days equals age approximately 6 months (following 200 days on the E- or E+ defined diets with 3500 mg ascorbic acid/kg diet or on the lab diet), the E- (open circle) and E+ (square) zebrafish were then switched to the low ascorbic acid (50 mg/kg) defined diets. At time 0, there were no significant differences in body weight (mean $\pm$ SD) between the groups (E-, E+ or lab); at 52 days on the diet regimen, the body weights of the lab diet fish (triangle) were greater ($P < 0.05$) than those of the defined diet fish, and by 145 days on diet all groups were different from each other (2-way ANOVA, time $p = 0.0276$, diet $p < 0.00001$, interaction, $P = 0.756$; $P < 0.05$; Tukey multiple comparisons; $n = 3/\text{diet group}$, except at the last point shown as 142 days (includes all fish harvested at 139 and 144 days): E- ($n = 32$), E+ ($n = 30$) and lab ($n = 30$) fish.

B. α -Tocopherol concentrations (nmol/g, mean $\pm$ SD) at 139 days in E- (open bars, $n = 10$), E+ (black bars, $n = 10$) and lab (striped bars, $n = 10$) zebrafish (ANOVA $P < 0.0001$, bars not bearing the same letter are significantly different $P < 0.05$, Tukey multiple comparisons).

C. Ascorbic acid concentrations (nmol/g, mean $\pm$ SD) in fish from each diet group ($n = 3/\text{group}$) were analyzed at days 0, 18, 34, 52; larger numbers of fish were analyzed at 139 ($n = 5/\text{group}$) and 144 days (E- $n = 6$, E+ or lab $n = 5/\text{group}$) (2-way ANOVA, time $p < 0.00001$, diet $p < 0.00001$, interaction, $p < 0.00001$). To assess changes over time, the data were logarithmically transformed and fitted by linear regression. The lab fish ascorbic acid concentrations did not change with time; ascorbic acid concentrations decreased faster in the E- compared with E+ fish (E-: $Y = -0.0207X + 5.4$, $R^2 = 0.9449$; E+: $Y = -0.0127X + 5.2$, $R^2 = 0.7841$; $P < 0.0001$). By days 139 and 144, the ascorbic acid concentrations were significantly different between all groups ($p < 0.05$, Tukey multiple comparisons).

D. MDA concentrations (nmol/g, mean $\pm$ SD) in E- ($n = 11$), E+ ($n = 10$), or lab ($n = 10$) zebrafish at 139 and 142 days (ANOVA $P < 0.0001$, bars not bearing the same letter are significantly different, $P < 0.05$, Tukey multiple comparisons).

Figure 2. Vitamin E deficiency causes impaired swimming behavior

The distances swam (cm, mean $\pm$ SEM) by E- (open bars, $n=31$) and E+ (filled bar, $n=30$) fish following stimulation with a single tap (A) or multiple taps (B); baseline measures are averages for each fish of three trials of 6 minutes without a stimulus. Following a single tap (A), the behaviors were different in E- and E+ fish (two-factor ANOVA, interaction $P=0.0028$); the E+ fish swam further at 1 minute than did the E- fish (#, $P<0.05$, Bonferroni post-hoc comparison), there were no other significant differences between the two groups at any of the time points. The E- fish at 1 minute swam farther than they did at 3, 5 or 6 minutes ($P<0.05$, Tukey's post-hoc comparison); the E+ fish swam farther at 1 minute than they did at all other time points ($P<0.05$, Tukey's post-hoc comparison); there were no other significant differences in distances swam. Following multiple taps (B), the behaviors were different in E- and E+ fish (two-factor ANOVA, interaction $P=0.0005$); at 1 minute the E+ fish swam farther than did the E- fish (#, $P<0.05$, Bonferroni post-hoc comparison), there were no other significant differences in distances swam between the two groups at any of the time points. The E- fish (open bars) swam farther at 1 minute than they did at all other time points; at 2 minutes they swam farther than at 4, 5 or 6 minutes ($P<0.05$, Tukey's post-hoc comparison). The E+ fish swam further at 1 minute than they did all other time points; at 2 minutes they swam further than at baseline, and all subsequent time points ($P<0.05$, Tukey's post-hoc comparison). For comparisons of single vs multiple taps (panel A vs B), both the E- fish (two-factor ANOVA, interaction $P<0.0001$) and the E+ fish (two-factor ANOVA, interaction $P<0.0001$) swam significantly farther at 1 and 2 minutes following multiple taps compared with the single tap ($P<0.05$, Tukey's post-hoc comparisons). Otherwise there were no significant differences in distances swam between the two levels of stimulation for each of the groups.

Figure 3. Vitamin E deficiency results in severe, polyphasic, degenerative myopathy

A. In E⁻ fish over 90% of the skeletal muscle is affected by progressive degenerative myopathy. Arrow heads indicate some of the remaining, atrophic myofibers widely spaced by edema and scattered inflammatory cells. Scales on the body surface are indicated by the thin arrow; a bone is visible in cross section near the bottom of the photo (arrow). H&E, 100 *X*, scale bar: 800 μ m. **B.** Myofibers from an E⁻ fish (shown in the bottom half of B) have loss of sarcoplasm and are collapsed (empty myotubes, arrow heads). This chronic myofiber degeneration is accompanied by mild interstitial edema. Histologically normal appearing myofibers are found on the top left. Also present is a diagonal area in the middle showing acute myofiber degeneration with loss of striation and fragmentation of sarcoplasm (arrows) that is accompanied by mild edema and loose infiltrates of mononuclear inflammatory cells. This polyphasic necrosis is typical of an ongoing degenerative process seen with chronic or repeat insult to skeletal muscle. H&E, 400 *X*, scale bar: 160 μ m. **C.** E⁺ fish have histologically normal muscle fibers. Scales on the body surface are indicated by the arrow. H&E, 100 *X*, scale bar: 800 μ m.

Figure 4. Increased oxidative damage in E- zebrafish muscles

Immunofluorescence employing an antibody to 4-hydroxynonenal (and a fluorescent secondary antibody) was used to assess muscle tissue damage caused by lipid peroxidation in E- and E+ zebrafish (n=5/group), as well as in one negative control fish. Slides were viewed/photographed under a Rhodamine filter. Fluorescence intensity was quantified using ImageProPlus (Media Cybernetics, Bethesda, MD). Representative photos show: **A.** E- fish. The intense fluorescent signal outlines myofibers of E- fish consistent with formation of 4-hydroxynonenal adduct in the sarcolemma. **B.** E+ fish. Staining of sarcolemma is minimal in E+ fish. **C.** Negative control. There is minimal background staining of the sarcoplasm and sarcolemma of the E- fish when the anti-HNE is omitted. **D.** Quantitation of fluorescence in E- and E+ zebrafish (n=5/group). Asterisk is p<0.05.