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Vitamin E and C supplementation reduces oxidative stress, improves antioxidant enzymes and positive muscle work in chronically loaded muscles of aged rats

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ABSTRACT

Aging is associated with increased oxidative stress. Muscle levels of oxidative stress are further elevated with exercise. The purpose of this study was to determine if dietary antioxidant supplementation would improve muscle function and cellular markers of oxidative stress in response to chronic repetitive loading in aging. The dorsiflexors of the left limb of aged and young adult Fischer 344 Brown × Norway rats were loaded 3 times weekly for 4.5 weeks using 80 maximal stretch–shortening contractions per session. The contra-lateral limb served as the intra-animal control. The rats were randomly assigned to a diet supplemented with Vitamin E and Vitamin C or normal non-supplemented rat chow. Biomarkers of oxidative stress were measured in the tibialis anterior muscle. Repetitive loading exercise increased maximal isometric force, negative work and positive work in the dorsiflexors of young adult rats. Only positive work increased in the aged animals that were supplemented with Vitamin E and C. Markers of oxidative stress (H_2O_2, total GSH, GSH/GSSG ratio, malondialdehyde and 8-OHdG) increased in the tibialis anterior muscles from aged and young adult animals with repetitive loading, but Vitamin E and C supplements attenuated this increase. MnSOD activity increased with supplementation in the young adult animals. CuZnSOD and catalase activity increased with supplementation in young adult and aged animals and GPx activity increased with exercise in the non-supplemented young adult and aged animals. The increased levels of endogenous antioxidant enzymes after Vitamin E and C supplementation appear to be regulated by post-transcriptional modifications that are affected differently by age, exercise, and supplementation. These data suggest that antioxidant supplementation improves indices of oxidative stress associated with repetitive loading exercise and aging and improves the positive work output of muscles in aged rodents.

1. Introduction

Aging causes deleterious modifications at genetic, cellular, tissue, and system levels in all organisms. The fundamental mechanisms of aging are poorly understood, but a growing body of evidence supports the idea that oxidative stress is an important contributing factor to the deterioration of organ and cell function that is associated with aging (Harman, 1956; Van Remmen et al., 2003; Gianni et al., 2004; Asano et al., 2007; Ryan et al., 2008).

The age-associated loss of skeletal muscle mass and strength (i.e. sarcopenia), is an unavoidable part of aging. Sarcopenia is likely mediated, at least in part, by a lifetime of damage from oxidants. Aging is associated with an increase in oxidant production and a decrease in the capacity to buffer oxidants, resulting in a chronic state of oxidative stress. Oxidative stress can damage biomolecules (DNA, lipids and proteins), decrease muscle protein synthesis, elevate apoptotic signaling and protein degradation (Gianni et al., 2004; Ryan et al., 2008). Although exercise is one approach that may counterbalance sarcopenia, oxidative stress that is developed during muscle contractions, may limit the ability of muscle from aged animals to undergo hypertrophy in response to exercise (Blough and Linderman, 2000; McArdle et al., 2002; Ryan et al., 2008).

Vitamin E (i.e., α-tocopherol) and Vitamin C (i.e., ascorbic acid) are antioxidants that are thought to have a protective effect by either reducing or preventing oxidative damage. Lipid soluble Vitamin E prevents lipid peroxidation chain reactions in cellular membranes by interfering with the propagation of lipid radicals. Vitamin C is a water-soluble antioxidant found in the cytosol and extracellular fluid that can interact directly with free radicals, thus preventing oxidative

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damage (Beyer, 1994). Due to their different subcellular locations, a combination of Vitamin E and C has been shown to have a better antioxidant effect than either of the two vitamins alone (Rokitzki et al., 1994; Rinne et al., 2000).

Oxidants generated near cellular membranes can oxidize Vitamin E forming a tocopheroxyl radical. Vitamin C may reduce the Vitamin E radical, thereby regenerating Vitamin E (Beyer, 1994; Halliwell, 1996). This reaction forms the semi-dihydroascorbate (Vitamin C radical), which in turn is reduced by a glutathione (GSH) (Rokitzki et al., 1994). Rodents (Chang et al., 2007) and humans (Bartali et al., 2008) that are deficient in Vitamin E show massive increases in pro-oxidant production and lipid peroxidation after exercise. Furthermore, low plasma concentrations of Vitamin E, associated with nutritional deficiencies often seen in the elderly (Leotsinidis et al., 2000; Guigoz et al., 2002), have been shown to be associated with a decline in physical function within these individuals (Bartali et al., 2008). In contrast, dietary supplementation of Vitamin E has been shown to increase tissue resistance to exercise-induced oxidative damage (Kumar et al., 1992; McBride et al., 1998; Itoh et al., 2000). In addition, recent data suggest that antioxidant supplementation can stimulate muscle protein synthesis in aged rats, possibly through the protection of leucine metabolism (Marzani et al., 2008). Furthermore, Vitamin E and C supplementation combined with resistance training has been shown to both increase fat free mass and muscle mass index in older adults, more than resistance training alone (Labonte et al., 2008).

Indicators of oxidative stress (lipid peroxidation measured by malondialdehyde (MDA)) have been shown to increase immediately after heavy resistance training in the plasma of humans (McBride et al., 1998). Plasma MDA levels returned to baseline in subjects who consumed a diet that was supplemented with Vitamin E, whereas plasma MDA levels continued to be elevated 24 h after resistance exercise in the non-supplemented subjects (McBride et al., 1998). However, this is not a universal finding because dietary supplementation with Vitamin E does not completely protect elderly men from oxidative damage caused by exercise (Alessio and Blasi, 1997). This may be due to low levels Vitamin C in the elderly, which could reduce the effectiveness of Vitamin E to protect against exercise-induced damage in the elderly (Alessio and Blasi, 1997). Thus, both Vitamins E and C may be important for effectively protecting muscles in aged people against oxidative damage.

Vitamin C is a highly effective water-soluble antioxidant primarily found in the cytosol and extracellular fluid. Even in small amounts, Vitamin C can protect proteins, lipids, carbohydrates, and nucleic acids from damage by pro-oxidants generated during normal metabolism. Vitamin E and glutathione also rely on Vitamin C for restoration back to their reduced isoforms. Vitamin C supplementation has been reported to have a protective effect against exercise-induced muscle damage (Jakeman and Maxwell, 1993). Though some studies have shown that taking antioxidants such as Vitamins E and C will prevent damage to tissues by reducing oxidant production, chronic use of these antioxidants could hinder the positive adaptive response that exercise has on the endogenous antioxidant defense system (Ristow et al., 2009). It is not clear, if Vitamin C has a direct role in muscle recovery from exercise, or if rather, it has an indirect role in this process.

Resistance training has been shown to be an effective means of increasing muscular size and strength, although the extent of the increase is attenuated with aging. However, repetitive mechanical resistant-type loading exacerbates oxidative stress in muscles of aged rodents, whereas it appears to be well buffered in muscles of young adult animals (Ryan et al., 2008). Oxidative stress increases in skeletal muscle after acute exercise (Ryan et al., 2010); however, chronic exercise enhances the endogenous antioxidant defenses and decreases production of pro-oxidants resulting in lower indices of oxidative stress. Previous data have shown that aging reduces the adaptive capacity of muscle to buffer the increased oxidant production imposed by chronic repetitive loading. The reduced buffering capacity may compromise the muscles' abilities to hypertrophy and/or to improve muscle function in aged animals.

Although more work is needed in this area, the combined data suggest that antioxidant supplementation may be a potential strategy for reducing exercise-induced oxidative stress and attenuating sarcopenia in the elderly. However, this is not a straightforward issue, because although Vitamin E and C supplementation will reduce oxidative stress post-exercise, the reduction in oxidative stress may inhibit redox sensitive pathways that are associated with the positive adaptation to exercise (Ristow et al., 2009).

Previous work suggests that there is a partial ability for oxidative enzymes to compensate for the increased oxidative insult in tibialis anterior muscles of aged rats in response to chronic repetitive loading as compared to young adult animals (Ryan et al., 2008). The current study tested the hypothesis that dietary supplementation with Vitamin E and C would lessen oxidant activity and oxidative damage in tibialis anterior muscles of aged rats subjected to chronic repetitive loading. Furthermore, in this study we assessed whether dietary supplementation with Vitamins E and C would attenuate the increase in basal levels of oxidative stress associated with aging, allowing for a more complete adaptation in oxidative enzymes, and improvements in muscle function after 4.5 weeks repetitive loading in the aged rats. The novel finding of this study was that antioxidant supplementation improved indices of oxidative stress associated with repetitive loading exercise and aging. Furthermore, antioxidant supplementation improved positive work in dorsiflexor muscles of aged animals.

2. Materials and methods

2.1. Experimental design

The dorsiflexors of the left limb of young (12 weeks of age; n = 14) and aged (30 months of age; n = 14) male Fischer 344 Brown × Norway rats were subjected to repetitive loading exercise. Seven animals from each age group were randomly assigned to a diet supplemented with Vitamin E (DL-alpha tocopheryl acetate; 30,000 mg/kg) and Vitamin C (L-ascorbic acid; 2% by weight) or normal non-supplemented (NS) rat chow containing 126 mg/kg of Vitamin E and 0% Vitamin C. All animals had free access to rat chow and water. The antioxidant or NS diets began seven days before the first exercise session. The non-supplemented animals were a subset of animals described in another study (Ryan et al., 2008). All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and fully conformed to the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings."

2.2. Muscle function

Maximal isometric muscle force, positive work, and negative work were assessed in the left exercised and right control limbs on a custom-built dynamometer (Cutlip et al., 2006). The dorsiflexor muscle group was activated indirectly through electrical stimulation of the common peroneal nerve via platinum stimulating electrodes (Grass Medical Instruments, Quincy MA, USA). Muscle stimulation for all protocols was via 120 Hz square wave pulses at 200 μs pulse duration, and 4 V. Dorsiflexor isometric force was measured at an ankle angle of 90°, using a stimulation duration of 300 ms. Positive and negative work was averaged from the first three individual stretch–shortening contractions in each exercise session (Cutlip et al., 2006). The stretch–shortening contraction was performed by activating the dorsiflexor muscles for 300 ms then moving the load cell fixture from 70° to 140° at an angular velocity of 500°/s. The load cell

fixture was immediately returned to 70°. Activation was continued for 300 ms after cessation of the movement.

2.3. Unilateral repetitive loading exercise

Repetitive loading consisted of 3 sessions per week for 4.5 weeks, with 80 stretch/shortening (i.e., eccentric/concentric) contraction cycles per exercise session (Cutlip et al., 2006). This method has been previously shown to produce a hypertrophic response in muscles of young adult rats (Cutlip et al., 2006) although aging attenuates the hypertrophic response to these loading conditions (Cutlip et al., 2006; Ryan et al., 2008).

2.4. Muscle preparation

Forty-eight hours after the last exercise session, the tibialis anterior of both loaded and control limbs were removed with the animal under anesthesia (2% isoflurane). The rats were then euthanized, via an overdose of ketamine/xylazine (30%/70%, v/v). The muscles were washed in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, and a pH of 7.4), blotted dry, and then weighed. A section of the muscle was obtained for the determination of the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). The remaining muscle was snap frozen in liquid nitrogen and stored at −80 °C.

2.5. RNA isolation

Sixty micrograms of frozen muscle was homogenized in 1 ml of Tri-Reagent (Molecular Research Center, Cincinnati, OH) with a motorized blade homogenizer. The RNA was isolated according to our standard procedures (Pistilli et al., 2006; Ryan et al., 2008). The RNA was treated with DNAse I using a DNA-free kit (Ambion, Austin, TX) and quantified using a Bio-Rad SmartSpec 3000. The RNA samples were quantified if their 260:280 ratios were 1.7 or greater.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Two micrograms of total RNA were reversed transcribed using 1.0 μl of random primers, 1.0 μl of 10 mM dNTP, and 1.0 μl of SuperScript II reverse transcriptase (Invitrogen/Life Technologies, Bethesda MD) as previously described (Ryan et al., 2008). The resulting complimentary DNA (cDNA) was stored at −80 °C or used for PCR analyses.

Primers for the genes of interest were designed as follows: CuZnSOD sense-5′-AGGCCGTGTGCGTGCTGA-3′; anti-sense-5′-CCCAATCA-CACCACAAGCCA-3′; GPx-1 sense-5′-CCTCGTGGCCTGGTGGTCCT-3′; anti-sense-5′-AGGGGTTGCTAGGCTGCTTGGA-3′; Mn SOD sense-5'-GCGGGGGCCATATCAATCACAG-3'; anti-sense-5'-GGCGGCAATCTG-TAAGCGACCT-3'; Catalase sense- 5'-CGGGAACCCAATAGGAGA-TAAA-3'; anti-sense-5'-CCACGAGGGTCACGAACTGT-3' as previously published by our lab (Ryan et al., 2008). Preliminary experiments were conducted to ensure that the number of PCR cycles were completed in the linear range of amplification for each gene of interest. PCR products were verified by restriction digestion based on predicted PCR sequences. Routine PCR amplification was conducted using PCR buffer, $MgCl_2$, 5 mM dNTPs, 100 ng/ml of primer pairs, 18S primer pairs, 1.0 μl of Taq DNA polymerase, and 1.0 μl of cDNA (Ryan et al., 2008). Amplification of PCR products were performed over a linear range of application cycles, using a thermocycler using: a denaturing step at 95 °C for 45 s, an annealing step for 45 s, and an extension step at 72 °C for 45 s. 20 μl of each PCR product was separated by electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide to visualize the PCR products. The signal from each PCR gene product was expressed as a ratio to the 18S signal from the same PCR product. The PCR signals were recorded via a digital camera (Kodak 290) and the signals were quantified in arbitrary units as optical density × band area, using 1D Kodak image analysis software (Eastman Kodak Company, Rochester, NY).

2.7. Muscle protein fractionation

Cytoplasmic and nuclear protein fractions were obtained from 75 mg of frozen tibialis anterior using methods as reported previously by our lab (Siu et al., 2005; Ryan et al., 2008). Muscle samples were homogenized in 500 μl of ice-cold lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 20 mM HEPES at pH 7.4, 20% glycerol, 0.1% Triton X-100, and 10 μM dithioreitol) with a mechanical homogenizer. A lower concentration of dithioreitol was used than in previous studies (Siu et al., 2005; Ryan et al., 2008), to prevent interference with subsequent enzyme activity assays. Muscle homogenates were centrifuged at 800 *g* for 5-minutes at 4 °C. The supernatants were collected and centrifuged three times at 3500 *g* for 5-minutes at 4 °C. The resulting supernatant was collected as the nuclei-free cytosolic fraction and divided into two equal portions; the first portion was frozen at −80 °C until needed, and a protease inhibitor cocktail containing 104 mM 4-[2-aminoethyl]-benzenesulfonylflouride hydrochloride (AEBSF), 0.8 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A and 1.4 mME-64 (Sigma-Aldrich, St. Louis, MO, USA) was added to the second portion before it was frozen at −80 °C. Protein concentrations for each sample were determined in triplicate via a DC protein concentration assay (Bio-Rad, Hercules, CA). The cytosolic fraction was used in the following assays: H_2O_2 concentration, catalase activity, GPx activity, CuZnSOD and MnSOD activity, and western immunoblots.

2.8. Western immunoblots

The protein contents of glutathione peroxidase-1 (GPx-1), catalase, copper–zinc superoxide dismutase, (CuZnSOD) and manganese superoxide dismutase (MnSOD) were measured in the cytosolic protein fractions. Thirty micrograms of protein was loaded into each well of a 4–12% gradient polyacrylamide gel (Novex, Invitrogen) and separated by routine SDS-polyacrylamide gel electrophoresis (PAGE) for 1.5 h at 20 °C and transferred to a nitrocellulose membrane. The membranes were blocked in 5% non-fat milk protein (NFM) for 1 h at room temperature then incubated in appropriate dilutions of primary antibodies (diluted in 1% NFM in Tris-buffered saline with 0.05% Tween-20 (TBS-T) overnight at 4 °C. The membranes were washed in TBS-T followed by incubation in appropriate dilutions of secondary antibodies (diluted in 5% NFM in TBS-T) that were conjugated to horseradish peroxidase. The protein signals were developed using a chemiluminescent substrate (ECL Advanced, Amersham Bioscience) and visualized by exposing the membranes to X-ray films (BioMax MS-1; Eastman Kodak). Digital records were captured by a Kodak 290 camera and protein bands quantified using 1-D analysis software (Eastman Kodak, USA). The bands were quantified as optical density (OD) × band area and expressed in arbitrary units.

2.9. Hydrogen peroxide (H_2O_2) levels

A commercially available H_2O_2 kit (#FLOH 100-3; Cell Technology, Mountain View, CA) was used to measure H_2O_2 in muscle homogenates. The assay was performed with several modifications to the manufacturer's directions. Briefly, 50 μl of controls, samples, or H_2O_2 dilutions were mixed with 50 μl of the reaction cocktail in each well to initiate the reaction. The plate was incubated in the dark for 10 min, at 20 °C and fluorescence was detected with an excitation at 530 nm and measured at 590 nm. All analyses were done in duplicate and the samples were normalized to muscle protein concentration in each sample via a DC protein concentration assay (Bio-Rad, Hercules, CA).

2.10. GSH and GSH/GSSG ratio

A commercially available kit (#21040, Percipio Biosciences, Inc., Burlingame, CA) was used to measure glutathione (GSH), and oxidized glutathione (GSSG) in muscle homogenates. The GSH/GSSG ratio was determined from these data. The assay was conducted with several modifications to the manufacturer's directions. Briefly, muscle tissue (~40 mg) was homogenized immediately after dissection in 530 µl cold 5% metaphosphoric acid (MPA) for the GSH sample and for the GSSG sample ~40 mg of muscle tissue was homogenized immediately after dissection in 500 µl cold 5% metaphosphoric acid (MPA) and 30 µl of M2VO scavenger. Homogenates were then frozen in liquid nitrogen and stored at −80 °C until analyzed.

The assay was conducted as described by the manufacturer. Briefly, cold 5% MPA was added to each sample mixed, and centrifuged at 1000 *g* for 10 min. Fifty microliters of sample and the appropriate buffer and 50 µl of 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) in $Na \cdot PO_4$ and 50 µl of glutathione reductase in $Na \cdot PO_4$ were mixed and incubated at room temperature. 50 µl of NADPH was added and the absorbance of each sample was read every 60 s at 412 nm for 3 min. The protein concentration for each sample was determined via a DC protein concentration assay (BIO RAD). Signals from each sample were normalized to the corresponding protein content of that sample.

2.11. Oxidative DNA damage as measured by 8-hydroxy-2′-deoxyguanosine (8-OHdG)

A commercially available enzyme linked immunoassay (#21026; Percipio Biosciences, Inc., Burlingame, CA) was used to measure oxidized DNA in isolated muscle DNA samples. DNA was extracted from the muscle via DNeasy Tissue Kit (Qiagen, Valencia, CA). DNA was used if it had a minimum 260:280 ratio of 1.8. The assay was performed with several modifications to the manufacturer's directions. Briefly, 50 µl DNA was incubated with the primary antibody, washed, and then incubated in secondary antibody. The chromogen (3,3′,5,5′-tetramethylbenzidine) was added to each well, and incubated at room temperature in the dark for 15 min. The reaction was terminated and the samples were read at an absorbance of 450 nm. Samples were normalized to the DNA concentration measured via a plate reader (ND-1000, NanoDrop, Wilmington, DE). All analyses were done in duplicate.

2.12. Lipid peroxidation

A commercially available kit (#21020, Percipio Biosciences, Inc., Burlingame, CA) was used to measure malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) in muscle homogenates. MDA + HAE was used as an indicator of lipid peroxidation in skeletal muscle samples. The assay was conducted with minor modifications from that recommended by the manufacturer. Briefly, ~100 mg of muscle was homogenized in ice-cold PBS, containing 5 µL 0.5 M butylated hydroxytoluene (BHT) in acetonitrile per 1 ml of tissue homogenate. The muscle homogenate was centrifuged at 3000 *g* at 4 °C and the supernatant was used for the assay and protein determination. The muscle sample was incubated in the appropriate reagents according to the manufacturer's instructions, and centrifuged at 15,000 *g*. An absorbance reading of the supernatant was obtained at 586 nm. Samples were normalized for differences in the amount of muscle protein in each sample as determined by a DC protein concentration assay (Bio-Rad, Hercules, CA).

2.13. Catalase activity

A commercially available catalase activity assay kit (#219265, EMD/Calbiochem, San Diego, CA), was used to measure catalase activity in muscle homogenates. The assay was conducted according to the manufacturer's recommendations. All analyses were completed in duplicate and samples were read at absorbance of 520 nm. The data were normalized to muscle protein in each sample via a DC protein concentration assay (Bio-Rad, Hercules, CA).

Table 1
Body mass of young and aged rats pre and post 14 sessions of repetitive loading. Data are mean ± SEM and presented as the mass of the rats before the first exercise session and after the 14th training session in grams.

	Young NS	Young Vit. E and C	Aged NS	Age Vit. E and C
Pre-RL (g)	343.5 ± 10.9	350.1 ± 26.1	513.8 ± 88.4†	491 ± 63.5†
Post-RL(g)	328.1 ± 11.8	340.2 ± 27.8	467.2 ± 91†	441 ± 55.5†

Each value expressed in grams as a mean ± SEM.
NS = non-supplemented diet.
† Indicates a significant difference ($p<0.05$) from young exercise and diet-matched control muscles.

2.14. Manganese superoxide dismutase (MnSOD) and copper–zinc superoxide dismutase (CuZnSOD)

A commercially available kit (#574601, EMD/Calbiochem, San Diego, CA) was used to measure total and MnSOD activity in muscle homogenates. CuZnSOD was calculated by subtracting the value for MnSOD activity from the total SOD activity. The assay was performed with minor modifications to the manufacturer's directions and all samples and standards were measured in duplicate. Briefly, the muscle was homogenized in 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose and centrifuged at 1000 *g* for 10 min. The assay was performed in a 96-well plate with each sample being treated with and without 10 µl of 12 mM potassium cyanide. Potassium cyanide was used to inhibit CuZnSOD, resulting in the detection of only MnSOD activity. The reagents and samples were protected from white light and incubated at 26 °C for 20 min with periodic shaking. The absorbance was measured at 450 nm using a 96-well plate reader (Dynex Tech., Chantilly VA., USA).

2.15. Glutathione peroxidase (GPx)

A commercially available cellular GPx assay (#35319, EMD/Calbiochem, San Diego, CA) was used to measure GPx activity in the cytosolic fractions of the muscle homogenates. The assay was performed with several modifications to the manufacturer's directions. Briefly, a portion of each muscle was homogenized in a buffer

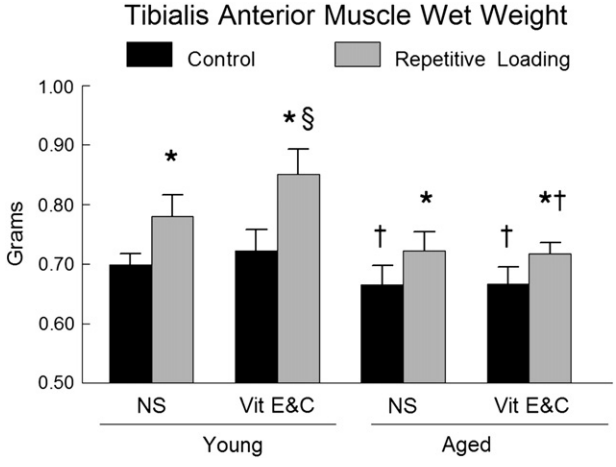

Fig. 1. Repetitive loading induces muscle hypertrophy in the tibialis anterior muscle. Tibialis anterior muscle wet weight is reported for young and aged rats that received no dietary supplement (NS) or a diet supplemented with Vitamin E and C (Vit E& C). Data are expressed as mean ± SEM. *, significant difference between age-matched control and repetitive loaded TA muscle assigned at $p<0.05$; †, a significant difference ($p<0.05$) from young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) from age-matched animals on the non-supplemented diet.

containing 50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1 mM DTT. The homogenate was centrifuged at 10,000 *g* for 15 min at 4 °C and the supernatant was used for the assay. All reagents and samples were equilibrated to 25 °C and the remaining assay procedures followed manufacturer's guidelines. The absorbance was measured at 340 nm using a 96-well plate reader (DYNEX Technologies, Chantilly Va., USA). Each sample and control was performed in duplicate.

2.16. Statistical analyses

Statistical analyses were performed using an SPSS 18.0 software package. Statistical significance of the data was calculated by a multiple analyses of variance (MANOVA). When significant F scores were indentified from the MANOVA, subsequent protected one-way analysis of variance followed by Tukey post-hoc tests were used to identify differences between means. Statistical significance was accepted at $p \leq 0.05$. Data are reported as mean ± standard error mean (SEM).

3. Results

3.1. Body weight and food consumption

The body weight characteristics of all of the animals examined in this study are shown in Table 1. The average body weight of the aged animals was ~49.5% ($p<0.05$) more than in the young animals. Bodyweight did not change over the course of the study in either the control or Vitamin E and C supplemented animals.

Food consumption was similar in old rats on the control and the antioxidant diets. The old rats consumed 20.3 ± 1.3 g of food per day in the control diet group and 20.8 ± 1.1 g in the antioxidant diet group. As a result, the old rats in the control diet consumed ~2.56 mg (~4.98 mg/kg bodyweight) of Vitamin E and 0 mg of Vitamin C/day. The rats in the supplemented diet group consumed ~624 mg (~127.1 mg/kg body weight) of Vitamin E and 412 mg (~84.7 mg/kg body weight) of Vitamin C per day.

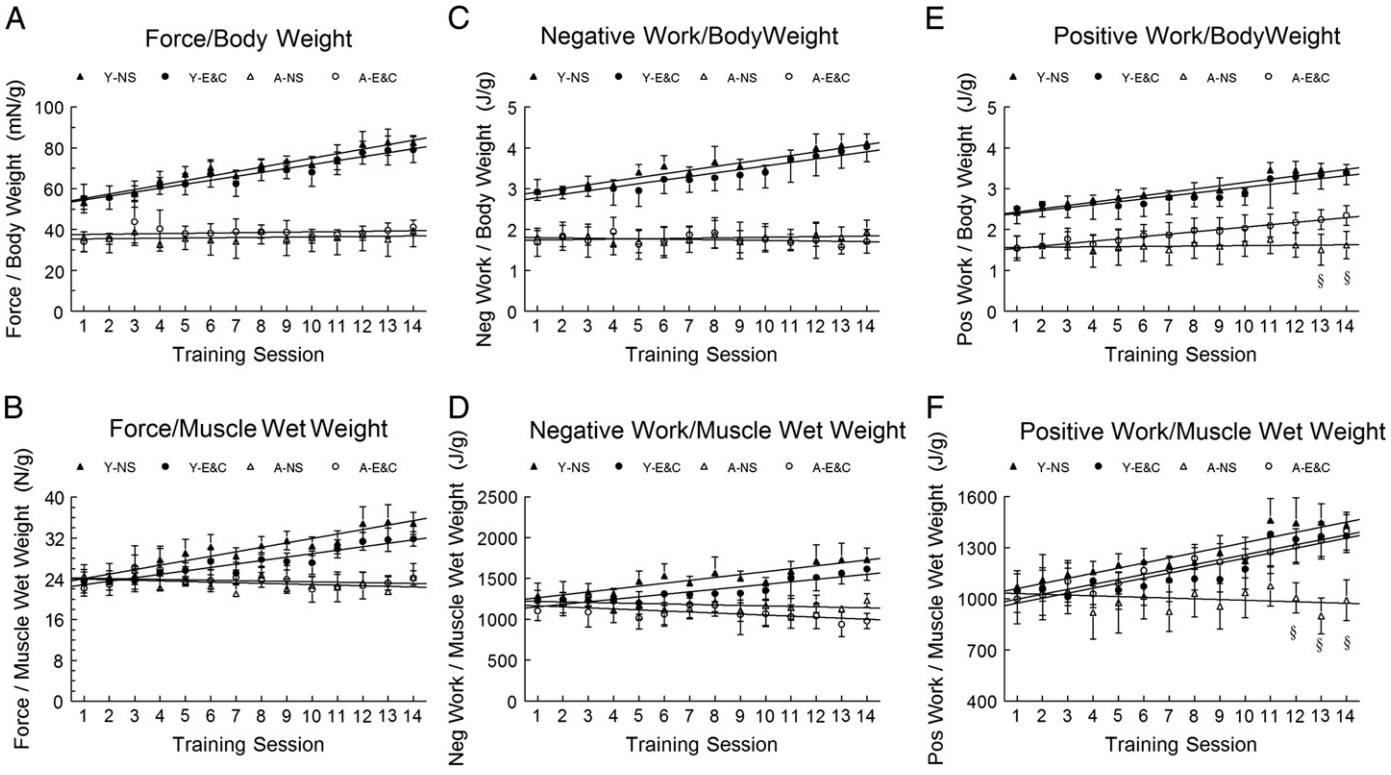

Fig. 2. Repetitive loading increased muscle functional measurements in young adult dorsiflexor muscles while maintaining function in aged dorsiflexor muscles. (A) Maximal isometric force generated in the dorsiflexors normalized to bodyweight from young and aged rats during each of the 14 training sessions. Data are expressed as the average maximum isometric force/body weight for all animals in milli-Newtons (mN) normalized to body mass (BM) in grams (g) produced during each exercise session ± SE. There was no significant difference in maximal dorsiflexor isometric force output between rats that consumed the control, or the antioxidant diet, for young adult or aged animals. The solid line represents the linear regression for all age-matched points. (B) Maximal isometric force generated in the dorsiflexors normalized to muscle wet weight from young and aged rats during each of the 14 training sessions. Data are expressed as the average maximum isometric force/body weight for all animals in Newtons (N) normalized to tibialis anterior muscle wet weight in grams (g), at the end of the study. Data are expressed as mean ± SE. The solid line represents the linear regression for all age-matched points. (C) Negative work generated from a single eccentric/concentric movement performed at the start of each training session from the young and aged dorsiflexor muscles during each of the 14 training sessions. The data are expressed as joules normalized to body weight (J/g). There was no significant differences between diets. The solid line represents the linear regression for all age-matched points. (D) Negative work generated from a single eccentric/concentric movement performed at the start of each training session from the young and aged dorsiflexor muscles during each of the 14 training sessions normalized to tibialis anterior wet weight at the end of the study. The data are expressed as joules normalized to tibialis anterior wet weight (J/g). The solid line represents the linear regression for all age-matched points. There was no significant differences between diets. (E) Positive work generated from a single eccentric/concentric movement performed at the start of each training session from the young and aged dorsiflexor muscles during each of the 14 training sessions. §, indicates that there was a significant difference ($p<0.05$) from age-matched animals on the non-supplemented (NS) diet. The data are expressed as joules normalized to body weight (J/g). The solid line represents the linear regression for all age-matched points. (F) Positive work generated from a single eccentric/concentric movement performed at the start of each training session from the young and aged dorsiflexor muscles during each of the 14 training sessions. Data are normalized to tibialis anterior wet weight obtained at the end of the study and they are expressed as J/g. The solid line represents the linear regression for all age-matched points. §, indicates that there was a significant difference ($p<0.05$) from age-matched animals on the non-supplemented (NS) diet. Data are expressed as the mean ± SEM. The solid line represents the linear regression for all age-matched points. Maximal isometric force, negative work and positive work generated from the young animals was significantly different ($p<0.05$) from aged dorsiflexor muscles at all time points.

3.2. Muscle wet weight

Exercise increased tibialis anterior muscle mass in aged animals, but there was no greater improvement in the accumulation of muscle mass in the rats receiving the antioxidant diet (Fig. 1). Exercise increased tibialis anterior muscle wet weight in the exercised limb of non-supplemented young adult (13.1%, $p<0.05$) and aged rats (7.5%, $p<0.05$), as compared with the contra-lateral control muscle. The wet weight of the tibialis anterior muscle increased with exercise in Vitamin E and C supplemented young adult rats (17.8%, $p<0.05$) and aged rats (7.7%, $p<0.05$), but this was not different from the adaptations of muscles in the non-supplemented animals.

3.3. Muscle function

Dorsiflexor muscle function was normalized to body weight (Fig. 2A, C, and E) and tibialis anterior wet weight (Fig. 2B, D, F) for young adult and aged rats. Maximal force, positive work and negative work were all greater in control non-exercised muscles and exercised muscles from young adult compared to aged rats. Exercise increased muscle function in both young adult groups but only positive work increased in the exercised dorsiflexors of aged rats. Maximal isometric force increased by 48% ($p<0.05$) in the exercised dorsiflexors of young adult non-supplemented animals and by 40% ($p<0.05$) in the dorsiflexors of Vitamin E and C supplemented young adult rats (Fig. 2A, B). Negative work was increased in the dorsiflexors from young adult non-supplemented rats (35.7%, $p<0.05$) and Vitamin E and C supplemented animals (31.9%, $p<0.05$). Vitamin E and C provided no additive improvement in negative work of young adult animals over exercise alone (Fig. 2C, D). Exercise increased positive work increased in the dorsiflexors of non-supplemented young adult animals (35.9%, $p<0.05$) and Vitamin E and C supplemented rats (30.6%, $p<0.05$) (Fig. 2E, F). Furthermore, positive work increased in both the control non-exercised muscles (40%, $p<0.05$) and the exercised dorsiflexors (37.7%, $p<0.05$) of aged rats that consumed the Vitamin E and C supplemented diet compared to non-supplemented animals (Fig. 2E, F). Compared to the non-supplemented diet, Vitamin E and C did not alter maximal force or negative work in the dorsiflexors of aged rats.

3.4. H_2O_2

The antioxidant diet prevented the exercise-induced increased muscle levels of H_2O_2 in muscles from both young adult and aged rats. Exercise increased the levels of H_2O_2 by 44.8% ($p<0.05$) in the tibialis anterior muscles of the non-supplemented animals. Although there was a 37.7% ($p<0.05$) increase in H_2O_2 in the exercised tibialis anterior of Vitamin E and C supplemented young adult rats, this was significantly less ($p<0.05$) than the increase of H_2O_2 in the muscles of non-supplemented animals (Fig. 3). Exercise increased the levels of H_2O_2 by 37.1% ($p<0.05$) in the tibialis anterior muscles of the non-supplemented aged animals. Vitamin E and C supplementation suppressed H_2O_2 in non-exercised muscles and prevented the exercise-induced increase in H_2O_2 in exercised tibialis anterior muscles from both young adult and aged rats (Fig. 3).

3.5. Total glutathione

Total glutathione was reduced with aging but increased with exercise in the tibialis anterior of aged animals. Total glutathione was 33% ($p<0.05$) lower in the non-exercised tibialis anterior muscles of aged as

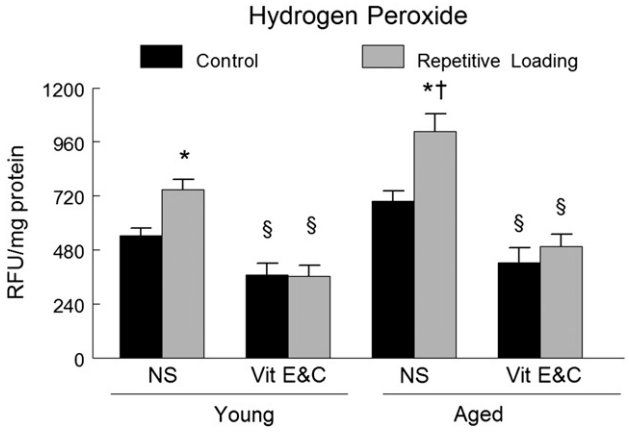

Fig. 3. Vitamin E and C supplementation attenuated the increase in hydrogen peroxide (H_2O_2) concentration associated with exercise and aging. The H_2O_2 concentration was determined fluorometrically. The data are expressed as mean ± SEM of relative fluorescent units (RFU) per mg of total protein homogenate. *, significant difference ($p<0.05$) between age-matched repetitively loaded muscle and contra-lateral control muscle; †, a significant difference ($p<0.05$) from young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) between age-matched muscles from animals on the non-supplemented (NS) diet.

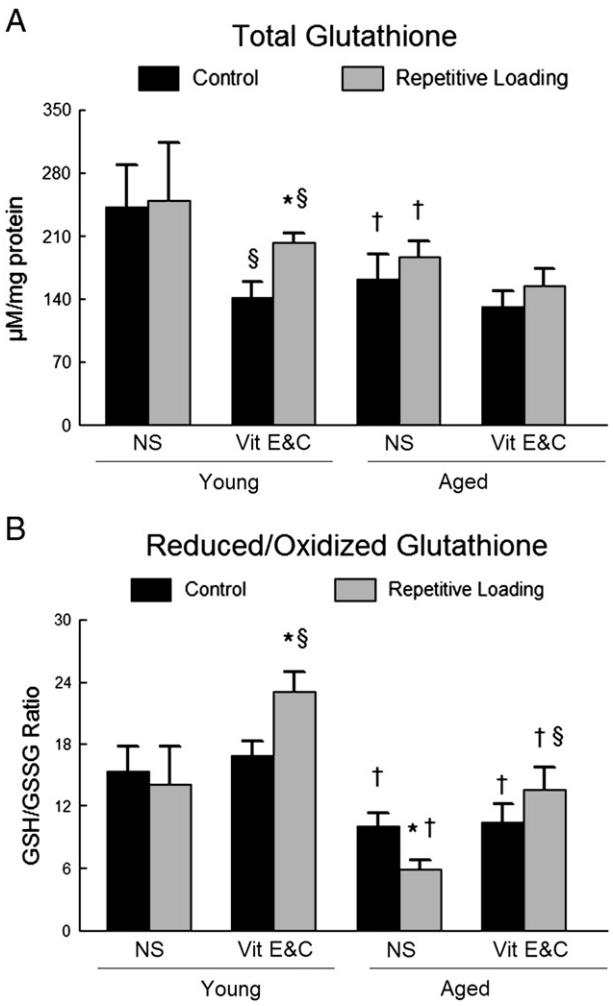

Fig. 4. Total glutathione content and the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG). (A) Data indicate total glutathione concentration normalized to total protein content. (B) Data are depicted as the ratio of GSH to GSSG normalized to total protein content. Lower ratios are an indication of increased oxidative stress. The normalized data are presented as mean ± SEM. *, significant difference ($p<0.05$) between age-matched repetitively loaded muscle and contra-lateral control muscle; †, a significant difference ($p<0.05$) from young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) between age-matched muscles from animals on the non-supplemented (NS) diet.

compared with young adult rats. Exercise training increased total glutathione by 43% ($p<0.05$) in the tibialis anterior muscles from young adult animals that were fed the Vitamin E and C supplemented diet (Fig. 4A). In contrast, Vitamin E and C supplementation did not alter total glutathione in control or exercised muscles from aged rats (Fig. 4A).

3.6. GSH/GSSG ratio

The GSH/GSSG ratio in the tibialis anterior was lowered with aging. This is indicative of an elevation of skeletal muscle oxidative stress with aging. GSH/GSSG was reduced even further with exercise ($p<0.05$), but the exercise-induced reduction in GSH/GSSG was prevented in aged rats fed the Vitamin E and C diet (Fig. 4B). The combination of Vitamin E and C supplementation and repetitive loading, increased the GSH/GSSG ratio in exercised muscles of young adult rats (36.1%, $p<0.05$), and aged rats (117%, $p<0.05$). Vitamin E and C supplementation did not improve GSH/GSSG in the unexercised control muscles from young adult or aged animals (Fig. 4B).

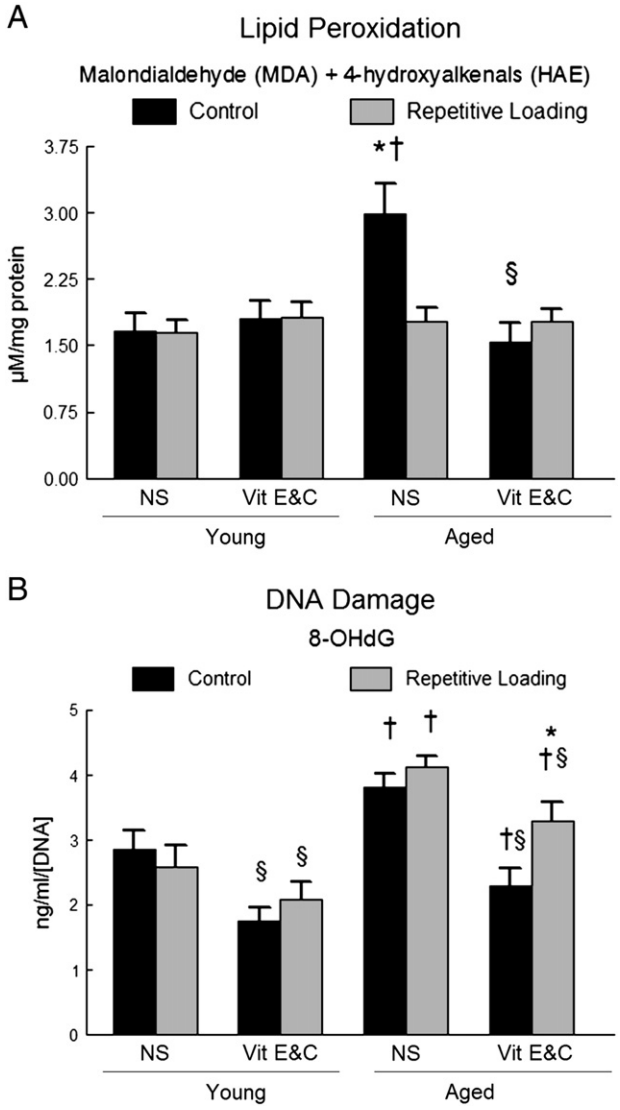

Fig. 5. Vitamin E and C supplementation decreased evidence of oxidative damage associated with repetitive loading exercise and aging. (A) The data represent oxidative damage as indicated by malondialdehyde (MDA) plus 4-hydroxyalkenals (HAE) normalized to total protein content. (B) Data are provided as mean ± SEM and they are expressed as the nanogram concentration of 8-hydroxy-2′-deoxyguanosine (8-OHdG) per ml of muscle homogenate per μg of DNA; *, significant difference ($p<0.05$) of repetitively loaded muscle from contra-lateral control muscle; †, a significant difference ($p<0.05$) from young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) from age-matched animals on the non-supplemented (NS) diet.

3.7. Lipid peroxidation

Oxidative stress, as indicated by lipid peroxidation (i.e., MDA + HAE), was greater in control and exercised muscles of aged rats than young adult rats, but Vitamin E and C suppressed exercise-induced lipid peroxidation in muscles of aged rats. MDA + HAE abundance was significantly greater in control muscles from aged rats (79.6%, $p<0.05$) as compared to young adults (Fig. 5A). Vitamin E and C and exercise reduced MDA + HAE in skeletal muscle of aged rats to levels that were not different from that measured in young adult rats. There was no additive effect of exercise and Vitamin E and C supplementation on the reduction of lipid peroxidation in muscles of aged rats (Fig. 5A).

3.8. DNA damage

8-OHdG was greater in muscles of aged animals than young adult animals, but this indicator of oxidative DNA damage was suppressed in muscles of animals that were supplemented by Vitamin E and C. 8-OHdG was significantly greater in control muscles from aged animals (37.2%, $p<0.05$) as compared to young adult animals (Fig. 5B). Vitamin E and C significantly lowered 8-OHdG in the muscles of young adult rats (−38.4%, $p<0.05$) and aged rats (−40.1%, $p<0.05$), as compared to non-supplemented animals of the same age. Although 8-OHdG was greater in exercised (56.6%, $p<0.05$), as compared to non-exercised muscles of aged Vitamin E and C supplemented animals, 8-OHdG was still lower (−20.4%, $p<0.05$) in supplemented animals than non-supplemented animals after exercise (Fig. 5B).

3.9. Glutathione peroxidase (GPx)

3.9.1. Enzyme activity

Gpx activity was increased with exercise but suppressed in exercised skeletal muscle of young adult and aged rats that consumed the Vitamin E and C diet. There was a loading effect, but no age effect, on muscle GPx activity. GPx activity was greater in exercised muscles of young adult (123%, $p<0.05$) and aged (71.8%, $p<0.05$) rats as compared to their contra-lateral non-exercised muscles (Fig. 6A). Vitamin E and C supplementation lowered total GPx activity in the tibialis anterior muscle of non-exercised (−60%, $p<0.05$) and exercised muscles (−194%, $p<0.05$) of young adult rats. Similarly, GPx activity was lower in non-exercised control muscles (−48%, $p<0.05$) and exercised muscles (−100%, $p<0.05$) of aged rats that consumed the Vitamin E and C diet as compared to the non-supplemented diet. Vitamin E and C blunted the exercise-associated increase in GPx in muscles from young adult and aged animals. There was no additive effect of Vitamin E and C supplementation and exercise in suppressing Gpx activity in either age group (Fig. 6A).

3.9.2. Gpx protein abundance

The muscle protein level of GPx-1 increased with Vitamin E and C supplementation in both young adult (265%, $p<0.05$) and aged (303%, $p<0.05$) animals. Exercise did not have a significant effect on GPx-1 protein concentrations in muscles of either age group (Fig. 6B).

3.9.3. Changes in GPx mRNA

Vitamin E and C suppressed GPx-1 mRNA expression in exercised and non-exercised muscles of aged rats. Vitamin E and C reduced Gpx-1 mRNA levels in non-exercised muscles from young rats (−41.8%, $p<0.05$) and in both exercised and non-exercised muscles from aged rats (−83.3%, $p<0.05$). Exercise did not alter GPx-1 mRNA levels in muscles of non-supplemented aged rats (Fig. 6C).

3.10. Catalase

3.10.1. Enzyme activity

Skeletal muscle catalase activity was increased by both aging and Vitamin E and C supplementation. Catalase activity was ~466% ($p<0.05$) higher in control muscles of aged vs. young adult animals (Fig. 7A). Vitamin E and C increased catalase activity in non-exercised control muscles from young (350%, $p<0.07$) and aged (147%, $p<0.07$) animals as compared to non-supplemented animals. Repetitive loading increased catalase activity in the aged Vitamin C and E supplemented animals (61.1%, $p<0.05$), but exercise did not alter catalase activity in muscles of non-supplemented young adult or aged rats.

3.10.2. Catalase protein abundance

The content of catalase protein was greater in muscles of aged as compared to young adult animals, and it was increased further with Vitamin E and C supplementation. Vitamin E and C supplementation increased catalase protein content in non-exercised muscles from young adult rats (60%, $p<0.05$) and aged rats (292%, $p<0.05$). There was a further increase in catalase protein content with exercise and Vitamin E and C supplementation in muscles of aged rats (246%, $p<0.05$), when compared to non-exercised muscles from Vitamin E and C supplemented aged rats (Fig. 7B).

3.10.3. Changes in catalase mRNA

Catalase mRNA was suppressed by both aging and Vitamin E and C. A diet high in Vitamin E and C diet reduced catalase mRNA levels in non-exercised (−24.7%, $p<0.05$) and exercised (−22.6%, $p<0.05$) muscles of young adult rats compared to the non-supplemented rats. Catalase mRNA expression was reduced in non-exercised (−44.4%, $p<0.05$) and exercised (−43.9%, $p<0.05$) muscles from Vitamin E and C treated aged animals (Fig. 7C). Exercise did not alter muscle catalase mRNA levels in any experimental group.

3.11. Copper–zinc superoxide dismutase (CuZnSOD)

3.11.1. Enzyme activity

Although exercise did not increase CuZnSOD activity in the muscles of aged rats, the exercise-induced increase in CuZnSOD activity that was seen in the muscles of young adult rats, was partially rescued in aged rats fed Vitamin E and C. Vitamin E and C increased CuZnSOD activity in the non-exercised muscles from young adult (255.6%, $p<0.05$) and aged (48.3%, $p<0.05$) animals. Exercise increased CuZnSOD enzyme activity in the muscles of non-supplemented young rats (64.1%, $p<0.05$) but not aged rats. Exercise further increased CuZnSOD activity in muscles of Vitamin E and C supplemented young adult (35.7%, $p<0.05$), and aged rats (20%, $p<0.05$), as compared to the non-exercised muscles in the supplemented group of animals (Fig. 8A).

3.11.2. CuZnSOD protein abundance

Vitamin E and C increased CuZnSOD protein content in the non-exercised muscles of aged animals. CuZnSOD protein abundance was 118% greater ($p<0.05$) in the non-exercised muscles from Vitamin E and C supplemented aged animals (Fig. 8B). Exercise increased CuZnSOD protein abundance in only the muscles of young adult rats (83.3%, $p<0.05$).

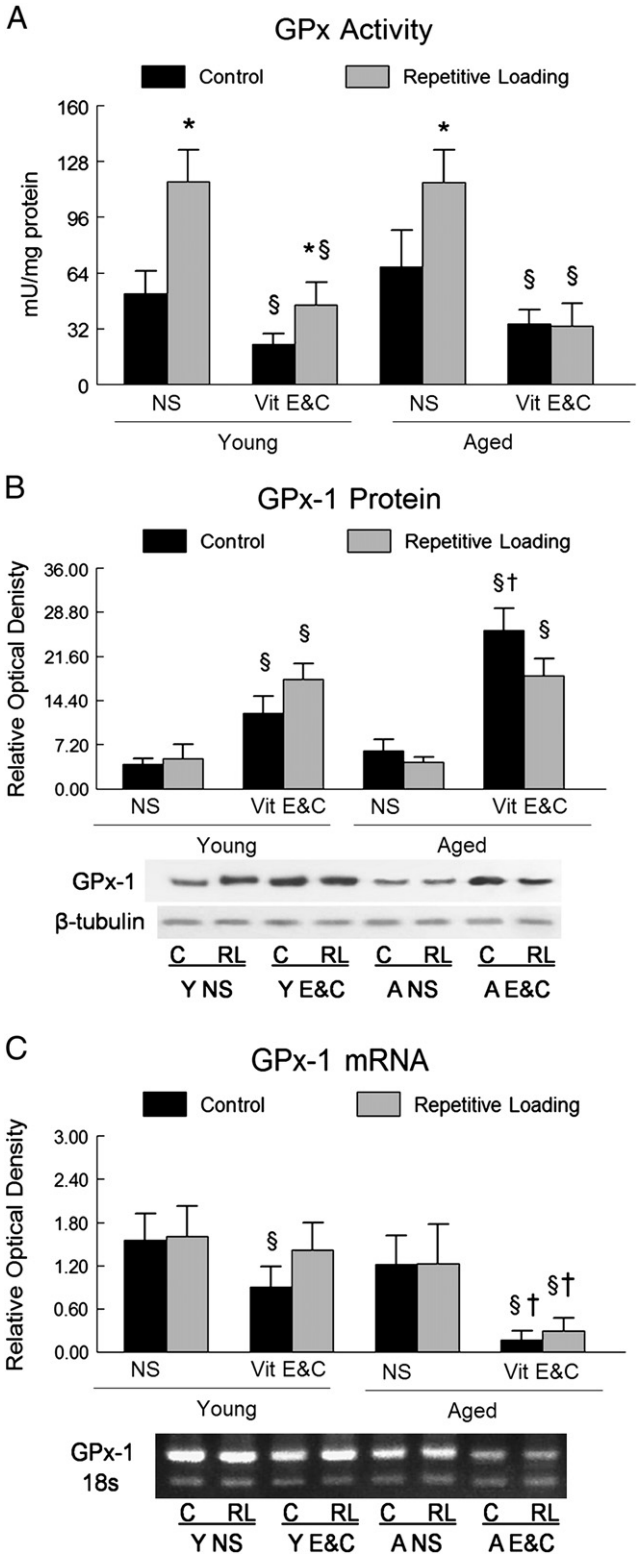

Fig. 6. Glutathione peroxidase (GPx) regulation with repetitive loading and Vitamin E and C supplementation. (A) Total GPx activity was expressed as mU of GPx per ml of muscle homogenate normalized per mg of protein in the homogenate. (B) GPx-1 protein expression was determined in the total cytosolic fraction by western immunoblots. The data are expressed as optical density (OD) × band area, and presented as relative optical density. The inserts show representative blots for GPx-1 and β-tubulin in young and aged (control and repetitive loading) tibialis anterior muscle. (C) GPx-1 mRNA expression was determined by RT-PCR. The data are expressed as optical density (OD) × band area, and presented as relative optical density. The inserts show representative gels for GPx-1 mRNA and 18s rRNA in young and aged (control and repetitively loaded) muscle. All data are presented as mean ± SEM; *, significant difference ($p<0.05$) between age-matched repetitively loaded muscle and contra-lateral control muscle; †, a significant difference ($p<0.05$) between young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) between age-matched animals on the non-supplemented (NS) diet; C, control; RL, repetitively loaded; Y, young; A, aged; E&C, diet supplemented with Vitamin E and C.

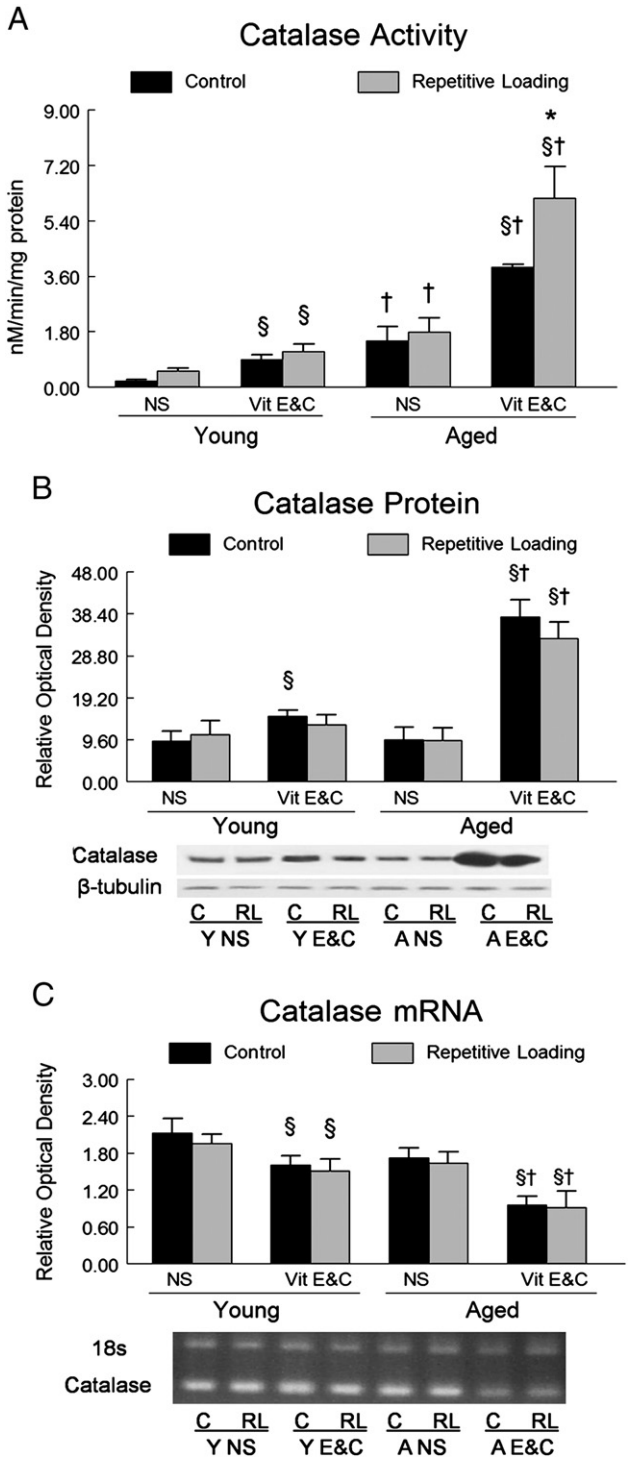

Fig. 7. Catalase regulation with repetitive loading and Vitamin E and C supplementation. (A) Catalase activity was determined at 520 nm and expressed as nM of catalase per ml of homogenate normalized per mg of protein in homogenate. (B) Catalase protein expression was determined in the total cytosolic fraction by western immunoblots. The data are expressed as optical density (OD) × band area, and presented as relative optical density. The inserts show representative blots for catalase and β-tubulin in young and aged (control and repetitively loaded) muscles. (C) Catalase mRNA expression was determined by RT-PCR. The data are expressed as optical density (OD) × band area, and presented as relative optical density. The inserts show representative gels for catalase mRNA and 18S rRNA in young and aged (control and repetitively loaded) muscles. All data are presented as mean ± SEM; *, significant difference ($p<0.05$) between age-matched repetitively loaded muscle and contralateral control muscle; †, a significant difference ($p<0.05$) between young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) between age-matched animals on the non-supplemented (NS) diet; E&C, diet supplemented with Vitamin E and Vitamin C; C, control; RL, repetitively loaded; Y, young; A, aged.

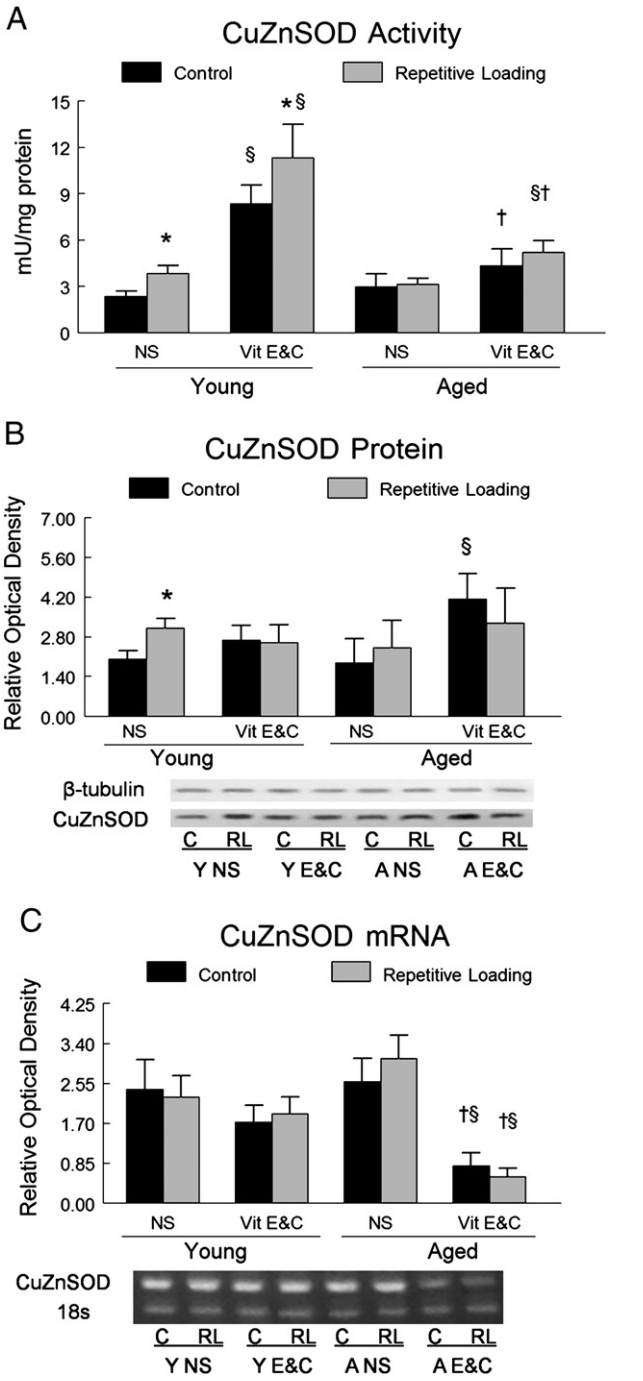

Fig. 8. CuZn superoxide dismutase (CuZnSOD) regulation with repetitive loading and Vitamin E and C supplementation. (A) CuZnSOD activity was expressed as mU of CuZnSOD per ml of homogenate normalized per mg of protein in homogenate. (B) CuZnSOD protein expression was determined in the total cytosolic fraction by western immunoblot. The data are expressed as optical density (OD) × band area, and presented as relative optical density. The inserts show representative blots for CuZnSOD and β-tubulin in young and aged (control and repetitive loading) TA muscle. (C) CuZnSOD mRNA expression was determined by RT-PCR. The data are expressed as optical density (OD) × band area, and presented as relative optical density. The inserts show representative gels for CuZnSOD mRNA and 18S rRNA in young and aged (control and repetitively loaded) muscle. All data are presented as mean ± SEM; *, significant difference ($p<0.05$) between age-matched repetitively loaded muscle and contralateral control muscle; †, a significant difference ($p<0.05$) between young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) between age-matched animals on the non-supplemented (NS) diet; E&C, diet supplemented with Vitamin E and Vitamin C; C, control; RL, repetitively loaded; Y, young; A, aged.

3.11.3. CuZnSOD mRNA

CuZnSOD mRNA content was suppressed by Vitamin E and C in aged muscles. CuZnSOD mRNA was reduced in both the non-exercised (−73.1%, $p<0.05$) and the exercised muscles (−82.9%, $p<0.05$) of aged rats in the Vitamin E and C supplemented group, compared to non-supplemented animals (Fig. 8C).

3.12. Manganese superoxide dismutase (MnSOD)

3.12.1. Enzyme activity

Vitamin E and C supplementation increased MnSOD activity levels in muscles of young adult animals. MnSOD activity was increased in the non-exercised muscles (200%, $p<0.05$) and in the exercised muscles (256%, $p<0.05$) from young adult animals that were fed the Vitamin E and C diet. Neither the Vitamin E and C diet, nor exercise altered MnSOD activity in the muscles from the aged animals (Fig. 9A).

3.12.2. MnSOD protein abundance

Vitamin E and C increased MnSOD protein abundance in non-exercised muscles of both young adult and aged rats. The Vitamin E and C diet increased MnSOD protein content in non-exercised muscles from young adult (180%, $p<0.05$) and aged animals (103%, $p<0.05$). Exercise increased MnSOD protein content in non-supplemented muscles from young adult animals (113%, $p<0.05$). However, MnSOD protein abundance was lower in the exercised muscles from Vitamin E and C supplemented young adult (−67.2%, $p<0.05$) and aged (−47.1%, $p<0.05$) rats, compared to the contra-lateral control muscles (Fig. 9B).

3.12.3. MnSOD mRNA

The increase in MnSOD protein content in Vitamin E and C supplemented muscles of aged rats, was not driven by increases in MnSOD mRNA expression. Rather, MnSOD mRNA expression was suppressed by the Vitamin E and C diet in both the non-exercised (−62.8%, $p<0.05$) and the exercised muscles (−63.3%, $p<0.05$) of aged rats (Fig. 9C). Exercise and the Vitamin E and C diet had no effect on MnSOD mRNA expression in control or exercised muscles of young adult animals.

4. Discussion

Oxidative damage has long been implicated in the aging process (Harman, 1956). Oxidative damage occurs in both type I and type II fiber types of aged animals (Fugere et al., 2006), and is thought to underlie at least part of the deterioration in skeletal muscle with aging (Bejma and Ji, 1999; Ryan et al., 2008). The aim of this study was to investigate the efficacy of dietary antioxidant supplementation to improve oxidative stress in skeletal muscle in aged rodents in response to repetitive loading. We chose to examine the tibialis anterior muscle, because it is composed of predominantly type II fibers, which are thought to be more susceptible to oxidative stress than type I fibers, because their antioxidant defenses are less extensive. It was hypothesized that dietary supplementation with Vitamin E and C would lessen the oxidant activity and oxidative damage in tibialis anterior muscles in an age-dependent manner. The sub-hypothesis was that Vitamin E and C supplementation would attenuate the increase in basal levels of oxidative stress associated with aging, allowing for improved adaptation in oxidative enzymes and muscle function after 4.5 weeks of repetitive loading in the aged

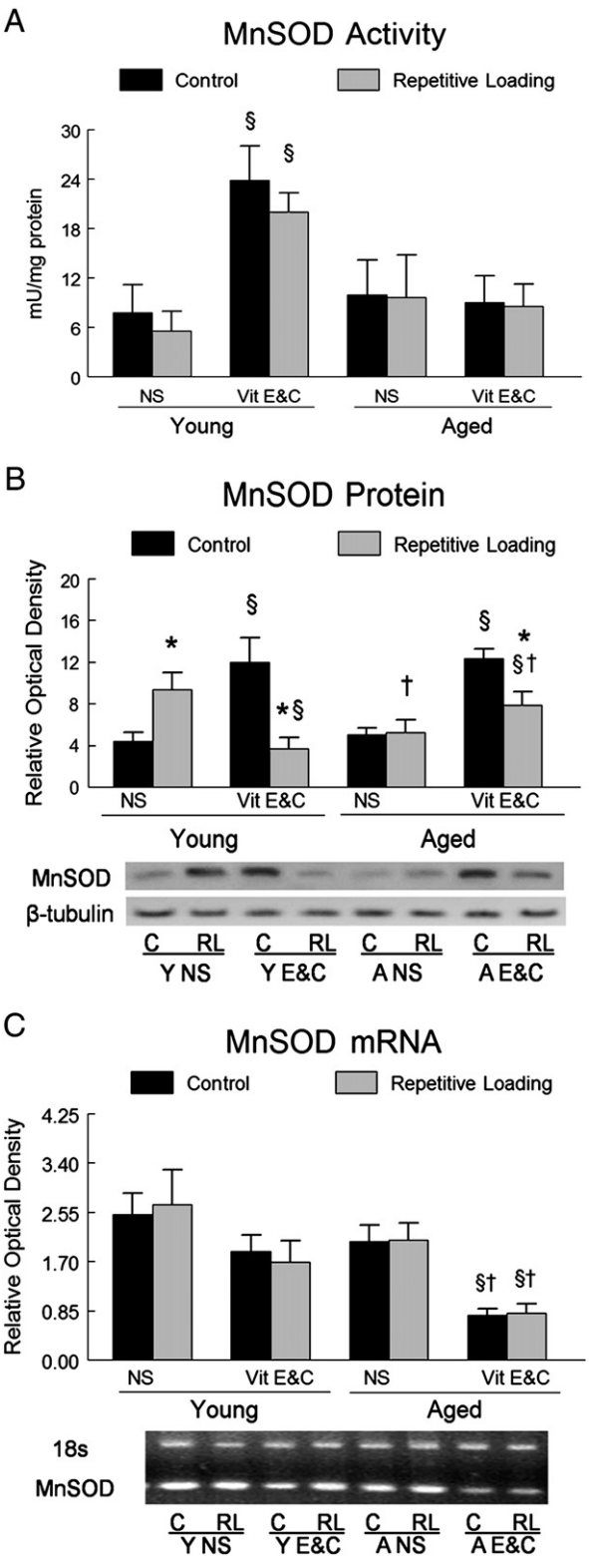

Fig. 9. Mn superoxide dismutase (MnSOD) regulation with repetitive loading and Vitamin E and C supplementation. (A) MnSOD activity was determined after inhibiting CuZnSOD activity by potassium cyanide. MnSOD was expressed as mU of MnSOD per ml of homogenate normalized per mg of protein in homogenate. (B) MnSOD protein expression was determined in the total cytosolic fraction by western immunoblot. The data are expressed as optical density (OD) × band area, and presented as relative optical density. The inserts show representative blots for MnSOD and β-tubulin in young and aged (control and repetitively loaded) muscle. (C) MnSOD mRNA expression was determined by RT-PCR. The data are expressed as optical density (OD) × band area, and presented as relative optical density. The inserts show representative gels for MnSOD mRNA and 18S rRNA in young and aged (control and repetitive loaded) muscle. All data are presented as mean ± SEM; *, significant difference ($p<0.05$) between age-matched repetitively loaded muscle and contra-lateral control muscle; †, a significant difference ($p<0.05$) between young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) between age-matched animals on the non-supplemented (NS) diet; E&C, diet supplemented with Vitamin E and Vitamin C; C, control; RL, repetitively loaded; Y, young; A, aged.

rats. The novel results of this investigation show that dietary supplementation with Vitamins E and C reduced oxidant levels in repetitively loaded muscles of aged rats, but there was no improvement in accumulation of muscle mass in the tibialis anterior muscle over 4.5 weeks of loading. Specifically, fortifying normal rat chow with Vitamin E and C, lowered the concentrations of H_2O_2, increased the ratio of GSH/GSSG, reduced indices of oxidative damage to DNA (8-OHdG) and cellular lipids (malondialdehyde and 4-hydroxyalkenals) in repetitively loaded tibialis anterior muscles from young adult and aged rats. Vitamin E and C supplementation did not improve maximal force production after more than four weeks of repetitive loading in muscles of aged animals. Interestingly, positive work output improved after exercise training in the dorsiflexors of aged animals that received the Vitamin E and C, but without increases in muscle wet weight or maximal force output.

4.1. Age-related adaptations of muscle weight to repetitive loading

Tibialis anterior muscle mass increased in response to 4.5 weeks of repetitive loading in aged rats (Ryan et al., 2008). However, in general, exercise-trained muscles in the aged animals only improved to the level of that observed in control untrained muscles of young adult animals. These results are consistent with previous observations in rodents using the same loading approach as the current study (Ryan et al., 2008), or other models of loading in rodents (Degens and Alway, 2003) and humans (Kosek et al., 2006), all showing that aging attenuates, but does not prevent muscle adaptation to loading until very old ages (Blough and Linderman, 2000; Raue et al., 2009). To our knowledge, this is the first report that has evaluated the aging-specific effects of repetitive resistance training in combination with Vitamin E and C supplementation on changes in muscle weight. Fortifying the diet with Vitamins E and C in an attempt to reduce oxidative stress during repetitive loading exercise did not provide any additive effect to improvements in muscle weight, maximal force production, or negative work output, in dorsiflexor muscles from young adult or aged rats. This implies that oxidative stress associated with aging may not be a limiting factor for protein accumulation and maximal force production in repetitively loaded muscles of aged animals. An alternative explanation is that the level of Vitamin E and C supplementation was too low to fully overcome any limitations to protein accumulation that occur in response to repetitive loading in muscles of aged rodents. Likely the antioxidant capacity of muscles in young adult animals was already sufficient to balance loading-induced increases in oxidative stress, so that elevating antioxidants via Vitamins E and C did not provide any greater improvement in muscle hypertrophy.

4.2. Age-dependent adaptations to antioxidant supplementation on muscle function after repetitive loading

Repetitive loading significantly increased maximal isometric dorsiflexor force production of single contractions by >40%, positive work (work performed during the concentric portion of each contraction) by >31%, and negative work (work performed during the eccentric portion of each contraction) by >30% in young adult rats. Positive and negative work were calculated at the beginning of each training session, so these represented non-fatigued contractions. The wet weight of repetitively loaded tibialis anterior muscles was not improved by the antioxidant supplementation, and since this muscle provides the greatest contribution to dorsiflexion, it is not surprising that Vitamin E and C supplementation did not further improve maximal dorsiflexion isometric force, positive or negative work in the dorsiflexors of young adult rats. In contrast, to the young adult rats, non-supplemented aged rats were unable to improve maximal force, positive or negative work over the training period. While Vitamin E and C supplementation did not improve maximal force or negative work, positive work was improved by ~38% in the aged rats that received the Vitamin E and C fortified diet. These findings indicate that although muscle size was not enhanced (and therefore maximal isometric force was not improved) by the antioxidant diet in the aged animals, the high oxidant environment of the aged muscle likely contributed to a rapid loss of force during each contraction in the non-supplemented animals, thereby affecting positive work output in each contraction. Vitamin E and C supplementation likely buffered (at least in part) the additional oxidant production imposed by repetitive loading, such that force was better maintained during each shortening contraction throughout the exercise session. It is possible that the antioxidant diet maintained force production over a single contraction better than the control diet, and this contributed to an improved ability to produce positive work in Vitamin E and C supplemented animals. Factors that contribute to a loss of contractile force include reactive oxygen species, which are mediators of muscle fatigue (Reid, 2008). It is possible that Vitamin E and C acts to lower basal levels of oxidative stress and therefore improves the redox environment during a single contraction at the beginning of the exercise session. This could reduce the drop in force over a single contraction, thereby improving positive work in muscles of aged animals.

4.3. Aging increases oxidative stress but Vitamin E and C reduces oxidative stress in loaded muscles

Several studies report an age-related increase of lipid peroxidation, oxidative modification to proteins, and DNA damage (Bejma and Ji, 1999; Gianni et al., 2004; Siu et al., 2008; Chen et al., 2008; Ryan et al., 2008). Similarly, the results of the current study suggest that the tibialis anterior muscles from aged rats are under greater oxidative stress than muscles from young adult rats. In addition to the age-associated increase in oxidative stress, repetitive loading exercise also elevated the oxidative load in skeletal muscles.

4.3.1. H_2O_2

The increase that we observed in cytosolic H_2O_2 content in the exercised tibialis anterior muscle is consistent with previous data showing that muscle contractions increase oxidant production (Davies et al., 1982; Kumar et al., 1992; McBride et al., 1998; Bejma and Ji, 1999; McArdle et al., 2005; Ryan et al., 2008, 2010). Hydrogen peroxide is a relatively stable pro-oxidant that in biological systems is most commonly produced from the dismutation of superoxide. It is commonly assumed that most of the increases in H_2O_2 during exercise, are the result of superoxide production.

In general, this study supports the idea that Vitamin E and C supplementation increases the ability of the muscle to buffer oxidant production associated with aging and exercise. To the authors' knowledge the current data are the first to show that Vitamin E and C supplementation directly lowers cytosolic H_2O_2 concentrations in both control and exercised tibialis anterior muscle from young adult and aged animals. It has been previously shown via electron spin resonance spectroscopy that Vitamin E supplementation lowers the concentrations of free radicals produced during 30 min of exhaustive swimming exercise, however this study did not further distinguish the type or location of radicals being produced (Kumar et al., 1992). Furthermore, no difference was reported in the muscles of non-exercised control animals (Kumar et al., 1992). The majority of the data that report a decrease in oxidant production after exercise with Vitamin E and C supplementation have measured oxidative damage to lipids, protein and DNA, but they generally have failed to measure oxidant production (Jakeman and Maxwell, 1993; McBride et al., 1998; Itoh et al., 2000; Rinne et al., 2000; Chang et al., 2007; Labonte et al., 2008). In the current study, Vitamin E and C supplementation lowered cytosolic H_2O_2 concentrations in the aged muscle to the same level as the young adult, and both the young adult and aged muscle from the supplemented animals were lower than the control limb of

the young adult non-supplemented tibialis anterior muscle. These data suggest that fortification of the rat's diet with Vitamins E and C was effective at increasing the oxidant buffering capacity, but combining exercise with supplementation did not increase further the buffering capacity of the tibialis anterior muscle to oxidative stress.

4.3.2. Glutathione

Total glutathione and especially, reduced glutathione (GSH) have important roles in protecting cells from oxidant damage. This protective function is achieved by direct conjugation with radicals as well as functioning as an electron donor in redox reactions. The redox reaction oxidizes GSH to GSSG while H_2O_2 and other peroxides are reduced (Maher, 2005). The ratio of reduced to oxidized glutathione (GSH/GSSG) is a good indicator of the redox status of the muscle. The results of this study show that aging decreased both the total GSH abundance and the GSH/GSSG ratio in skeletal muscle. This suggests a reduced potential for buffering oxidative stress in aged muscles. In addition, the GSH/GSSG ratio was lowered further in repetitively loaded muscles of non-supplemented aged rats, as compared to muscles in young adult animals. This indicates that aging reduced the ability to tolerate increased oxidative stress in chronically loaded skeletal muscles.

Interestingly, total GSH was lower in muscles from Vitamin E and C supplemented young adult rats as compared to non-supplemented animals. In contrast, Vitamin E and C supplemented aged rats did not reduce skeletal muscle GSH abundance further. Nevertheless, Vitamin E and C supplementation increased the GSH/GSSG ratio in the both the young adult and aged tibialis anterior muscle after chronic repetitive loading exercise. These data are consistent with the H_2O_2 data, and together this is indicative of increases in oxidant production during exercise. These data suggest that aging increased oxidative stress and therefore lowered the GSH/GSSG ratio as compared to muscles in young adult animals and that aging reduced that ability to tolerate increased oxidative stress in chronically loaded skeletal muscles. Furthermore, Vitamin E and C supplementation provided an effective buffer against oxidant stress in response to loading. It is not clear why the decrease in cytosolic H_2O_2 concentrations did not increase the GSH/GSSG ratio of non-exercised tibialis anterior muscle from young adult or aged animals receiving Vitamin E and C supplementation.

4.3.3. Oxidative damage to DNA

Oxidative DNA damage (8-OHdG) increased with aging but not with repetitive loading exercise. This is consistent with previous data, which indicate that aging is associated with increases in oxidative DNA damage (Radak et al., 2002; Sacheck et al., 2003; Ryan et al., 2008). Furthermore, in general, long-term exercise does not appear to elevate oxidative damage to DNA (Sacheck et al., 2003; Ryan et al., 2008), although the mode, duration and intensity of the exercise along with sampling procedures, may play a significant role in determining the effect that chronic exercise has on increased oxidative DNA damage in skeletal muscle. For example, in contrast to our current study using repetitive loading as a model of resistance exercise, 8 weeks of treadmill running resulted in an attenuation of the age-associated increase in 8-OHdG levels, and increased the activity of DNA repair in aged rats (Radak et al., 2002). It is likely that the differences in these studies are the result of the difference in the mode of exercise (high intensity, low duration vs. low intensity, high duration). Furthermore, chronic aerobic exercise has been shown to increase the mitochondria's oxidant buffering capacity and reduce oxidant production via the mitochondrial electron transport chain, whereas chronic resistance training has not been shown to elicit the same degree of adaptation within the mitochondria. It is also important to note that mitochondrial DNA is more susceptible to oxidative damage than nuclear DNA, so aerobic exercise-induced changes of oxidative stress may provide significant protection to mitochondria DNA, whereas this would likely not occur with resistance types of exercise. In the current study, Vitamin E and C supplementation decreased the 8-OHdG content in both young adult and aged muscle. This suggests that the antioxidant diet had a profound effect on buffering and reducing oxidative stress, resulting in lower DNA damage in muscles of both young adult and aged animals. Previous work has shown that Vitamin E supplementation decreased 8-OHdG in muscle of young men but supplementation failed to show a similar effect in elderly men (Sacheck et al., 2003). The variability of the subjects' heath/dietary status might explain some of the variability in the different responses of subjects to antioxidant supplementation.

4.3.4. Lipids

In contrast to DNA, the current data suggest that the increase in cellular damage to lipids associated with aging, can be attenuated after chronic repetitive loading. The beneficial effects of exercise on lowering levels of lipid peroxidation, has been observed in previous investigations (Alessio and Blasi, 1997; McBride et al., 1998; Ryan et al., 2008). The current study shows evidence that Vitamin E and C supplementation is as effective at reducing the elevated concentrations of MDA and HAE associated with aging as chronic repetitive loading alone. The current data is in agreement with previous work showing that aging increases oxidative stress (Bejma and Ji, 1999; Ji 2007; Asano et al., 2007) and that Vitamin supplementation (especially Vitamin E) protects lipids from oxidative damage (Kumar et al., 1992) observed during exercise and aging. However, there was not an additive effect of combining exercise with Vitamin E and C supplementation.

4.5. Adaptation of antioxidant systems to repetitive loading and antioxidant supplementation

The current investigation measured transcription, protein levels, and enzyme activity levels of endogenous antioxidant enzymes catalase, glutathione peroxidase, MnSOD, and CuZnSOD. Overall, the results do not support the likelihood that transcriptional control is a mechanism leading to increases in activity of the endogenous antioxidant enzymes. Instead, these data are consistent with the previously suggested notion that the endogenous antioxidant enzyme activities are regulated via various levels of post-transcriptional and/or post-translational controls (Hollander et al., 2000; Ryan et al., 2008). However, the current data do not support the view that Vitamin E and C supplementation inhibits the positive adaptations to exercise within all of the endogenous antioxidant enzymes (Ristow et al., 2009). The data in the current study suggest that there is an age-dependent effect of repetitive loading and Vitamin E and C supplementation within the tibialis anterior muscle. Vitamin E and C supplementation increased activity levels of catalase, MnSOD and CuZnSOD in both the exercise and control limbs from the young adult animals. There was no change in MnSOD activity but CuZnSOD and catalase also increased in the muscles of aged rats supplemented with Vitamin E and C. Although this not a universal finding (Gomez-Cabrera et al., 2008; Ristow et al., 2009), our data are consistent with reports that antioxidant vitamin supplementation increases the activities of the enzymatic antioxidants in both healthy (Shireen et al., 2008), as well as diseased animals with chronic elevations in oxidative stress.

Antioxidant supplementation has been previously reported to have a detrimental effect on producing expected antioxidant adaptations to chronic training (Gomez-Cabrera et al., 2008; Ristow et al., 2009). However, any detrimental effect may be due in part to the method of administering the supplement. For example, antioxidant supplementation after chronic training that is provided in a concentrated format (oral gavage or pill) likely induces a bolus effect and may reduce intestinal absorption. Whereas, in this study, rather than one mega dose, we provided the fortification of the animal's food, which, should produce more constant systemic levels of Vitamin E and

C (Giray et al., 2003). It is not currently known how the endogenous antioxidants enzyme levels would respond to the long-term effects of a diet fortified with Vitamin E and C taken throughout a period of chronic exercise, as compared to a single supplemented dose of Vitamin E and C that would be given after adaptation to chronic exercise.

5. Conclusions and limitations

The current study provides data to show that chronic exercise and Vitamin E and C supplementation lower indices of oxidative damage (i.e. 8-OHdG, MDA and HAE) associated with aging. However, repetitive exercise loading and Vitamin E and C supplementation affected DNA damage and lipid peroxidation differently. This raises the possibility that aging and loading types of exercise may increase oxidant production via different mechanisms. Several potential mechanisms exist that include elevations of oxidative stress via neutrophils and other infiltrating immune cells, mitochondria respiration, NADPH oxidase or xanthine oxidase activity. This possibility warrants additional studies, because previous work has suggested that aging increases oxidant production via the mitochondrial electron transport chain whereas exercise-induced increase in muscle oxidants originate from multiple sources (Bejma and Ji, 1999).

Assessing the effectiveness of Vitamin E and C supplementation in preventing exercise-induced oxidative stress has been difficult to determine from previous studies. This is the result of a wide variance in study designs and experimental conditions (i.e. subject/animal species, experimental conditions, length of study, dose of supplement, means of supplement administration and mode of exercise). Nevertheless, the data in our current study clearly show that Vitamin E and C supplementation lessens the oxidant activity and oxidative damage in tibialis anterior muscles from young and aged rats subjected to chronic repetitive loading. Furthermore, Vitamin E and C supplementation attenuated basal levels of oxidative stress associated with aging. Muscle size and other functional measurements were unaffected by Vitamin E and C supplementation, in aged rats, but there were important improvements in positive work in the aged animals after 4.5 week of repetitive loading that received the dietary supplementation.

A limitation of this study is that Vitamin E and C levels were not measured in the skeletal muscles of the experimental animals. Nevertheless, it is reasonable to assume that Vitamin E and C levels did change in the muscles of the treated animals, because muscle markers for oxidative stress was lower in the animals that consumed the antioxidant diets as compared to the animals that consumed the control diet.

It is probable that Vitamin E and C supplementation had effects other than those that were examined in skeletal muscles in the current study. As it was beyond the scope of this study, we did not measure central adaptations, systemic effects, or effects on other organs that would have been likely to occur by the antioxidant diet. Instead, this study focused on the local effects of Vitamin E and C on exercised muscles.

Antioxidant supplements may have differential effects on oxidative stress responses to exercise, depending on the timing, dose and duration of the antioxidant consumption (reviewed in Peake et al., 2007). In addition, the bioavailability of the supplement may also affect the magnitude of the results. For example, adaptations to exercise may have been improved if D-α-tocopherol was used in the current study, instead of the synthetic DL-α-tocopherol. This is because the bioavailability of the synthetic form Vitamin E is lower than its' natural form (Peake et al., 2007). Furthermore, although plasma levels increase rapidly after a bolus ingestion of Vitamin C or E, it has been suggested that it may take ~two weeks of treatment to fully saturate plasma levels of ascorbic acid and α-tocopherol, at least in humans (Levine et al., 1996; Meydani et al., 1997). While we began the dietary intervention one week before the first exercise session, we cannot rule out the possibility that a longer pre-treatment (e.g., for two weeks) with the dietary supplement may have improved the muscle adaptations to exercise. Although we did not measure plasma or muscle levels of Vitamin C or E, we would expect that the 4.5 week duration of the training period added to the one week of pre-treatment (a total of 5. 5 weeks of dietary intervention), would have been sufficient to obtain the maximal levels of the dietary supplements at the level of the muscles. Nevertheless, because the 5.5 weeks of Vitamin E and C diet did not improve muscle force production or muscle mass beyond the levels that were obtained in the control diet group, we believe that it is unlikely that an additional one week of pre-treatment would have substantially changed these findings. Furthermore, we would anticipate that consuming Vitamins E and C by diet would provide more constant levels of the antioxidants in plasma or muscle, as compared to studies that provided the supplements as a bolus. This is because ingestion of high levels of antioxidants are cleared rapidly by the kidneys and result in subsequent decreases in plasma levels within several hours of bolus ingestion (Levine et al., 1996).

Additional studies are required to determine if skeletal muscles in elderly humans who supplement their diet with Vitamin E and C during chronic resistance types of muscle loading, will respond in a similar fashion to the rodents in the current study. Currently, there are no data to indicate that dietary supplementation with Vitamins E or Vitamin C alters mortality, or the incidence of cancer in the elderly (Bjelakovic et al., 2008). Nevertheless, more global studies on the use of specific antioxidants and combinations of antioxidants to reduce oxidative stress in elderly humans are also warranted, given that meta-analysis (Bjelakovic et al., 2008) has suggested that some antioxidant supplements (e.g., Vitamin A, beta carotene and selenium) may be associated with increased mortality.

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