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ORIGINAL ARTICLE

Effect of 5-day vitamin E supplementation on muscle injury after downhill running in rats

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Abstract Antioxidant supplementation has been suggested to prevent exercise-induced muscle injury, but the findings are inconsistent. The objective of this study was to investigate the potential protective role of vitamin E treatment against eccentric exercise-induced muscle injury by examining morphological and functional alterations in rat soleus muscle after downhill running as well as muscle injury markers in the blood. Sixty adult male Wistar rats were randomly assigned to vitamin E-treated or placebo-treated groups studied at rest, immediately post-exercise or 48 h post-exercise ($n = 10$ per group). Vitamin E was administered by daily intraperitoneal injections of 100 mg/kg body mass of DL-α-tocopheryl acetate for five consecutive days prior to exercise, resulting in the doubling of its plasma concentration. Downhill running resulted in significant ($P < 0.05$) changes in all injury markers for the placebo-treated rats at 0 and 48 h post-exercise. However, significantly smaller soleus muscle single-twitch tension (P_t) and unfused (40 Hz) tetanic force, and greater plasma creatine kinase (CK) and lactate dehydrogenase (LD) activities compared with the control were found only immediately post-exercise for the vitamin E-treated rats ($P < 0.05$). Maximal tetanic force (P_o) did not decline significantly compared to sedentary controls at neither time points measured. The vitamin E-treated rats had significantly ($P < 0.05$) higher soleus muscle P_t immediately post-exercise than the placebo-treated rats as well as lower plasma CK and LD activity 48 h post-exercise. However, there was no difference in P_o decline between groups at either time points measured. Vitamin E-treated rats had less pronounced morphological alterations in muscle in the immediate and 48-h post-exercise period. In conclusion, the effect of short-term vitamin E supplementation against eccentric exercise-induced muscle injury did not appear to be physiologically significant, because vitamin E failed to prevent the decline in the functional measure of P_o compared to the placebo conditions.

Keywords Antioxidants · Eccentric exercise · Muscle damage · Contractile properties · Histology · Creatine kinase · Lactate dehydrogenase

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Introduction

Strenuous or unaccustomed physical activity is associated with skeletal muscle injury impairing physical performance

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(Clarkson and Hubal 2002). The extent of injury is greater when exercise involves eccentric (lengthening) muscle contractions rather than concentric or isometric contractions (Armstrong et al. 1983; Newham et al. 1983). Muscle injury is assessed through biochemical, morphological and functional alterations of the muscles involved. Markers of injury include structural changes in the histological appearance of the myofibres, muscle force deficit and increased concentration of myofibre proteins in the circulation (Warren et al. 1999).

Over the past two decades several nutritional supplements have been tested in humans and animals as potential interventions to alleviate exercise-induced skeletal muscle injury and its detrimental effects on physical performance (Bloomer 2007). A nutritional antioxidant commonly used to combat the injury and oxidative stress associated with exercise is vitamin E (Jackson et al. 2004; Sacheck and Blumberg 2001). The potential protective role of vitamin E against exercise-induced muscle injury and oxidative stress is attributed to its generalized and systemic effects as a potent chain breaking antioxidant (Burton and Ingold 1989) and stabilizer of membrane structure (Erin et al. 1984). Vitamin E is considered to be a major antioxidant, by scavenging free radicals that may harm membrane lipids (Burton and Ingold 1989), and a membrane stabilizer (Urano et al. 1993), by being embedded in the membrane bilayer, interacting with phospholipids and increasing the orderliness of membrane lipid packing (Erin et al. 1984). Vitamin E may thus contribute to sarcolemma integrity and, hence, protect muscle fibres from rupture during lengthening contraction.

Human and animal studies on the protective effects of vitamin E against skeletal muscle injury induced by eccentric contraction-biased exercise have yielded ambivalent and inconsistent findings. Early human studies implementing various exercise modalities, namely, box-stepping (Jakeman and Maxwell 1993), downhill running (Petersen et al. 2001), and eccentric elbow (Phillips et al. 2003) and knee (Beaton et al. 2002) contractions reported no apparent protection of vitamin E against skeletal muscle injury. However, more recent human studies also implementing downhill running (Sacheck et al. 2003), eccentric elbow (Bloomer et al. 2004) and knee (Shafat et al. 2004) contractions have provided some evidence that vitamin E may attenuate eccentric contraction-induced skeletal muscle injury.

Likewise, the data of the two animal studies found in the literature on the protective effects of vitamin E against eccentric exercise-induced muscle injury are also contradictory (Van der Meulen et al. 1997; Warren et al. 1992). While the former reported partial protection with vitamin E supplementation, the latter found no attenuation in any of the muscle injury markers determined. Warren et al. (1992) employed a downhill running protocol of relatively long duration and studied the slow twitch soleus muscle after feeding rats with a vitamin E-enriched diet, whereas Van der Meulen et al. (1997) applied an in situ lengthening contraction protocol to the fast twitch extensor digitorum longus muscle after injecting rats intravenously with vitamin E. The discrepancy in the findings observed in both human and animal settings may be partially attributed to the different experimental designs, exercise protocols and vitamin E supplementation regimes implemented in the studies.

The equivocal findings of the aforementioned studies suggest that a consensus is yet to be reached as to whether vitamin E can effectively protect skeletal muscle from injury induced by eccentric contraction-biased exercise. Further comprehensive research assessing diverse muscle injury markers, including neuromuscular measures, may be helpful in delineating possible sites of the effect of vitamin E supplementation. In addition to maximal tetanic force characterized by prolonged and maximal calcium ion release from the sarcoplasmic reticulum, the measurement of other contractile properties that reflect Ca^{2+} kinetics in the muscle may be useful. For example, single-twitch force and unfused tetanic force characterized by transient and limited Ca^{2+} release may prove more appropriate markers than maximal tetanic force in investigating exercise-induced muscle injury at the microtrauma level and test the ability of vitamin E to prevent injury. This particular element has not been investigated in previous studies.

Therefore, the aim of the present study was to explore the potential role of vitamin E treatment in attenuating skeletal muscle injury using biochemical, morphological and functional markers. Another objective was to explore the possibility of including particularly markers of Ca^{2+} kinetics in the muscle for the overall and more comprehensive assessment of exercise-induced muscle damage. To this end, a well-defined animal model was used: rats were initially treated with vitamin E and subsequently ran downhill on a treadmill. Unlike in situ and in vitro lengthening contraction protocols for inducing muscle damage, downhill running seems to simulate better the eccentric component of muscle contractions which take place in the actual exercise. Muscle injury was assessed by examining muscle injury markers in blood as well as morphological and functional alterations in soleus muscle.

Methods

Animals

The project was reviewed and approved by the institutional review board and the appropriate state authority. All

procedures were in accordance with the European Union guidelines for the care and use of laboratory animals, as well as the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985). Sixty adult (8–10 weeks old) male Wistar rats, weighing 220–270 g, were used in the study. The animals were housed under a 12 h light:12 h dark cycle, controlled temperature (18–21°C) and controlled humidity (50–70%). Commercial rat chow and tap water were provided ad libitum.

Rats were randomly divided into six groups as follows: placebo-treated sedentary control (Pb–Sed), placebo-treated and studied immediately after exercise (Pb–Ex0), placebo-treated and studied 48 h after exercise (Pb–Ex48), vitamin E-treated sedentary control (VE–Sed), vitamin E-treated and studied immediately after exercise (VE–Ex0) and vitamin E-treated and studied 48 h after exercise (VE–Ex48).

We decided to examine muscle injury at 0 and 48 h post-exercise because previous research has shown that skeletal muscle injury induced by downhill running is more apparent 2 days post-exercise compared to the typical sampling time point immediately post-exercise (Armstrong et al. 1983). In addition, the time points 0 and 48 h post-exercise highlight the two main stages (i.e. initial/autogenetic and phagocytic) of the four-stage muscle injury process, as proposed by Armstrong (1990). After the initial event that triggers the injury process, in our case the mechanical component of eccentric contraction-induced muscle injury, the succeeding autogenetic stage reflects primarily the initiation of degrading cellular structures via Ca^{2+}-activated proteolytic and lipolytic events. During the phagocytic stage, injury is mainly attributed to the metabolic events associated with the production of reactive oxygen species, the breakdown of the injured myofibrils and the removal of fibre debris. The phagocytic stage marked by a typical inflammatory response in the tissue is prevalent at 48–72 h post-exercise or even longer (Armstrong 1990). However, these stages of exercise-induced muscle injury should not be seen as separate distinct phases, each characterized by discrete physiological events, but rather as a continuum in which the physiological events are gradually transited and partially overlapped.

Supplementation

The animals of the vitamin E-supplemented exercised groups and those of the sedentary control group were injected intraperitoneally (i.p.) with 100 mg/kg body mass of DL-α-tocopheryl acetate per day for 5 consecutive days prior to exercise or examination, respectively (Kovacheva and Ribarov 1995; Hidalgo et al. 1988). Vitamin E was diluted in grain oil at a concentration of 4% (w/v). Accordingly, the placebo groups were injected with vehicle. This vitamin E regime was chosen because similar ones have previously increased vitamin E concentration in muscle (Van Der Meulen et al. 1997) and lung (Kovacheva and Ribarov 1995) and inhibited lipid peroxidation in the lungs (Kovacheva and Ribarov 1995) and the liver (Hidalgo et al. 1988). Furthermore, our rationale was to adopt a vitamin E administration regime in rats that approximates the doses most frequently used in human studies at the lower end of the supplementation period (i.e. approximately 1 week) and dosage (i.e. approximately 20 times the normal concentration) used in humans. This vitamin E supplementation approach seems to be more realistic and rational compared to long-term mega-doses of vitamin E supplemented in other studies.

According to the rat chow manufacturer (ELVIZ, Plati, Greece), the vitamin E content of the rat diet was 100 mg/kg chow. Given that the daily food consumption of an adult laboratory rat in the size range of 220–270 g is 10–20 g, it follows that a typical daily vitamin E intake would be 1–2 mg. We assume that this was the approximate daily vitamin E intake of the placebo-treated rats. In addition to that, the vitamin E-treated rats received an extra vitamin E dose of 100 mg/kg body mass daily, or 22–27 mg for rats weighing 220–270 g. This is approximately 15–20 times the vitamin E intake through the normal diet.

Exercise protocol

Exercise was performed after an overnight fast and 16–18 h after the last injection and consisted of 90 min of intermittent downhill running on a motor-driven treadmill. After a few minutes of familiarization with the procedure, the animals performed 18 bouts of 5-min running at a speed of 16 m/min down a 16° inclination, with a 2 min rest between bouts. Exercise of all animals was overseen by the same two experienced investigators. A detailed exercise log was kept for each rat, and animals from different groups were exercised in one exercise session. When necessary, their tails were stimulated to run using a soft-bristle brush. A couple of rats were excluded from the study, since they did not run as expected. After exercise, the animals of the Pb–Ex48 and VE–Ex48 groups were placed back in their cages with free access to food and water until they were examined. The exercise protocol implemented in this study has been widely used in previous studies and induced injury in the soleus and other rat hindlimb muscles (Armstrong et al. 1983; Ogilvie et al. 1988; Sotiriadou et al. 2006; Takekura et al. 2001). This study focused on the soleus, because it had been reported that downhill running induced more muscle injury to the soleus than other muscles in previous studies (Armstrong et al. 1983; Lynch et al. 1997; Smith et al. 1997). Furthermore, soleus is anatomically well-defined, easily dissected and easily prepared for in situ contractile property recordings. Given that it is composed of predominantly

(>85%) slow twitch fibres, soleus muscle is a sound model to study slow twitch muscle fibre damage in response to eccentric contraction-biased exercise (Armstrong and Phelps 1984).

Biochemical analysis

Blood samples were collected either at rest (16–18 h after the last injection) or immediately after exercise or 48 h following exercise as per group as follows. Under deep anaesthesia with chloral hydrate (4.5%, 1 ml/100 g body mass i.p.), the right front area of the neck was shaved and cleaned lightly with 70% ethanol. The skin was then sectioned and gently reflected using blunt-tip forceps to expose the right jugular vein from which about 1 ml of blood was drawn using a heparin-treated syringe. Blood was centrifuged at 3,000×*g* for 10 min and the resulting plasma was collected in Eppendorf vials, snap frozen in liquid nitrogen and stored at −80°C until analysis. Creatine kinase (CK) and lactate dehydrogenase (LD) activities were determined spectrophotometrically using kits from Randox (Crumlin, Co. Antrim, UK) according to the recommendations of the German Society for Clinical Chemistry. In a subset of three animals from each group, vitamin E was determined using high-performance liquid chromatography (Jasco HPLC System, Great Dunmow, UK) according to Nieremberg and Nann (1992). Each assay was run in duplicate and the inter-assay coefficient of variation (CV) was less than 10%.

In situ isometric tension recording

Following blood sampling, in situ isometric tension of the soleus muscle was recorded as follows. Under the same anaesthesia as above, the hair of the lower limbs was shaved up to the knee joint and the area was cleaned lightly with 70% ethanol. The skin was then sectioned and a longitudinal incision was made on the lateral surface of the right hindlimb over the area covered by gluteus superficialis. Adjacent muscles were gently reflected using blunt-tip forceps to expose the sciatic nerve. Likewise, a small incision was made on the back of the ankle, thus uncovering the distal (Achilles) tendon. The gastrocnemius muscle was retracted carefully to avoid rupturing the blood vessels, and the intact soleus muscle was exposed. The tendon was then tied with 3/0 silk thread and cut distally.

The rat was then placed prone on a stable rodent surgery table and was prepared for tension recording. Steel pins were inserted through the knee and ankle joints to stabilize the hindlimb, and cloth tape was used to secure the foot perpendicularly to the lower leg. Pins were supported with magnetic stand holders throughout the experiment. The tendon of the soleus muscle was attached to a strain-gauge transducer (UFI, Morro Bay, CA) by short silk suture, and bipolar silver electrodes were placed under the sciatic nerve, which was held in a relaxed position. Isometric contractions were evoked by stimulating the sciatic nerve (Digitimer DS9A stimulator, Hertfordshire, UK) using supramaximal (3–8 V) square pulses of 0.5 ms. Tetanic twitch stimulation was set at 350 ms. The signal from the transducer was amplified by a DC amplifier (Neurolog NL 107, Digitimer, Hertfordshire, UK), displayed on an oscilloscope screen (Fluke PM 3380A, Everett, WA), stored in a computer and processed using data acquisition software (FlukeView combiscope software, Everett, WA). A pulse programmer (Digitimer D4030, Hertfordshire, UK) controlled all devices during tension recording.

The muscle was adjusted to the optimal length (L_o) using a micromanipulator (Prior Scientific, Rockland, MA) allowing motion in all three directions. L_o was defined as the muscle length at which maximal twitch tension was obtained. It took 5–6 single-twitch trials to set L_o. Once this was set, a 1-min resting period was allowed before the actual recordings. Throughout the recordings, the longitudinal axis of the muscle remained aligned to the longitudinal axis of the transducer, and both remained parallel to the tibia.

The contractile properties that were measured were maximal isometric single-twitch tension (P_t), time-to-peak tension (TPT), single-twitch half-relaxation time ($RT_{1/2}$), isometric unfused tetanic force at 40 Hz and isometric maximal (fused) tetanic force (P_o) achieved at 80 Hz.

Throughout tension recording, the rat was kept warm with a heating pad. A diffuse heat source was also placed in close proximity to the surgery table. The depth of anaesthesia was assured by the constriction of the pupils as well as simple sensory tests, such as the absence of eye blinking when the eyelid was touched and the absence of foot withdrawal when the foot was pinched. When necessary, anaesthesia was maintained by administering approximately 10% of the initial dose. The sciatic nerve and soleus muscle were kept moist by periodically sprinkling Krebs solution at 37°C. Immediately after tension recording, the soleus muscle was excised and weighed on an electronic scale with an accuracy of 0.001 g. Animals were then euthanized by an overdose of chloral hydrate injected intravenously. Normalization of the data from muscles of different sizes was achieved by expressing generated force per muscle mass.

Muscle histology and histochemistry

Immediately following tension recording, the soleus muscle of the contralateral (left) hindlimb was carefully excised using 6 out of the 10 animals from each group and was attached to a wooden rod by pins inserted through the

tendon attachments so that the muscle remained elongated without being stretched. A sample was then excised from the midbelly and divided into two pieces: one for Harris's haematoxylin and eosin (HE) stain on frozen cross sections and the other for toluidine blue stain on longitudinal sections. In preparation for HE stain, the first muscle piece was mounted on a specimen holder so that muscle fibres were perpendicular to the holder, covered with a TissueTek optimum-cutting-temperature mounting medium (Sakura Finetek, Torrance, CA), frozen in liquid nitrogen-cooled isopentane and stored at −80°C. For analysis, frozen cross sections (8 μm) from the specimen were cut using a microtome cryostat (Zeiss Microm HM 505E, Heidelberg, Germany) and put onto Superfrost Plus glass slides (Erie Scientific, Portsmouth, NH). In preparation for toluidine blue stain, the second muscle piece was cut into smaller pieces of about 1 mm^3, fixed in phosphate-buffered 4% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded baths of ethanol and embedded in Epon epoxy resin (Serva, Heidelberg, Germany) with the fibres oriented longitudinally. For analysis, semi-thin longitudinal sections (1 μm) were cut from the Epon preparation with glass knives on ultramicrotome (Om U2, Reichert, Austria), put onto Superfrost Plus glass slides (Erie Scientific, Portsmouth, NH) and stained with toluidine blue. Toluidine blue stain was performed only on specimens obtained 48 h post-exercise, as it is at this time point of the muscle injury process that the phagocytic stage, marked by inflammatory response in the muscle, is prevalent (Armstrong 1990).

The evaluation of morphological alterations in soleus muscle following downhill running were performed according to Komulainen et al. (1994). From each muscle sample, five consecutive sections for either HE or toluidine blue stain were studied. A total of at least 300 myofibres from each rat were analysed. The image of the sections were projected on to the screen of a demonstration microscope and magnified to a constant size. Initially, muscle sections were examined in a magnification that enabled the entire section to be viewed to ensure that staining was consistent and no sectioning or staining artefacts were present. Microscope illumination intensity, once determined, then remained constant for analysis of all muscle sections. For each rat, 5 consecutive sections were cut and for each section one photo frame of magnification 40× (in each frame about 60 fibres can be visualized) was obtained from different parts of the section. Thus, all five photo frames cover a significant area of the section, yet the muscle fibres analysed do not cover the entire soleus muscle. The lesion densities/percentages were calculated by dividing the number of affected fibres counted by the total number of fibres analysed. The lesion densities/percentages obtained from each section were pooled to calculate the mean values. Edges of the sections were avoided and not included in the calculations because of the potential damage which occurred there during muscle dissection and subsequent processing. All sections were evaluated under a light microscope in a blind fashion by two experienced physicians specialized in pathology and anatomy. The evaluators recorded the frequency of the main pathological findings and the extent of damage in the exercised rats as compared to the non-exercised rats which had undergone the same treatment (placebo or vitamin E). The observations of the two evaluators were averaged.

Statistical analysis

Data were analysed with the SPSS, version 12, software (SPSS, Chicago, IL) and presented as mean ± SD. The distribution of all dependent variables (except for the morphological ones) was examined by the Shapiro–Wilk test and was found not to differ significantly from normal. To evaluate the potential protective effects of vitamin E supplementation against exercise-induced muscle injury, we performed 2 × 3 (supplementation by time) ANOVA followed by simple main effect analysis. The statistical significance level was set at $\alpha = 0.05$.

To determine the meaningfulness of the effects of exercise and vitamin E supplementation on muscle damage markers, effect sizes were calculated for each dependent variable as the difference between post- and pre-exercise mean values divided by the standard deviation of the pre-exercise value. Furthermore, the overall sensitivity of statistical power analysis was calculated for a total sample of 60 rats (6 groups of 10 rats) at 0.05 alpha level and 80% beta level (power) using the software G*Power 3 (Faul et al. 2007). The resultant effect size was 0.49 which is considered to be moderate according to both the original Cohen's scale (0.2 for small, 0.5 for moderate and 0.8 for large effects) (Cohen 1998) and the modified Cohen's scale (0.2 for small, 0.6 for moderate, 1.2 for large, 2.0 for very large and 4.0 for nearly perfect effects) (http://newstats.org). Correlation analysis between histological alterations of the soleus muscle and all other injury measures (i.e. muscle function and blood markers) determined at 48 h post-downhill running was assessed using the nonparametric Spearman's rank correlation coefficient. As described in "Methods", histological analysis was performed in six animals of each group only. Therefore, to increase the statistical power for the purpose of correlation, values of the placebo-treated Pb–Ex48 and vitamin E-treated VE–Ex48 groups were pooled together. We decided to perform correlation analysis on the samples taken at 48 h post-exercise because previous research has shown that peak manifestation of histological changes induced by downhill running occurs 2 days after the cessation of exercise.

Results

Plasma vitamin E concentration

Vitamin E supplementation doubled the circulating vitamin E concentration in the sedentary groups (from 9.3 ± 0.4 to 18.5 ± 3.3 mg/l, range 8.9–9.7 vs. 15.9–22.2 mg/l). In addition, the vitamin E-treated exercised rats had consistently higher plasma vitamin E levels compared to the placebo-treated exercised rats: immediately after exercise, 12.3 ± 0.7 mg/l (range 11.8–13.1 mg/l) versus 7.4 ± 2.3 mg/l (range 5.9–10.1 mg/l) and, at 48 h post-exercise, 13.8 ± 1.1 (range 13.0–15.0 mg/l) versus 10.2 ± 1.3 mg/l (range 8.8–11.3 mg/l). No statistical comparison was performed on these values because of the small sample size used for the particular analysis ($n = 3$ per group).

Plasma CK and LD activities

There was a significant supplementation-by-time interaction and significant main effects of supplementation and time on both enzymes ($P < 0.001$). Compared to the Pb–Sed group, the Pb–Ex0 and Pb–Ex48 groups showed significantly ($P < 0.001$) higher plasma CK and LD activities (Fig. 1), suggesting that the exercise protocol implemented induced skeletal muscle injury. Compared to the Pb–Ex0 group, CK activity was significantly lower, whereas LD activity was significantly higher in the Pb–Ex48 group ($P < 0.001$). In the vitamin E groups, CK and LD activities also increased immediately after exercise ($P < 0.001$) but returned to baseline 48 h post-exercise. Both enzymes were significantly ($P < 0.001$) lower in the VE–Ex48 group as compared to the Pb–Ex48 group. The effect sizes for CK in the placebo groups at 0 and 48 h were 5.59 and 3.67, respectively, whereas the corresponding effect sizes in the vitamin E groups were 6.99 and 3.67. The effect sizes for LD in the placebo groups at 0 and 48 h were 5.30 and 7.96, respectively, whereas the corresponding effect sizes in the vitamin E groups were 6.65 and 1.18. Overall, these data suggest that vitamin E supplementation did not prevent the increase in these enzymes immediately after exercise, yet it was effective in decreasing them 48 h post-exercise.

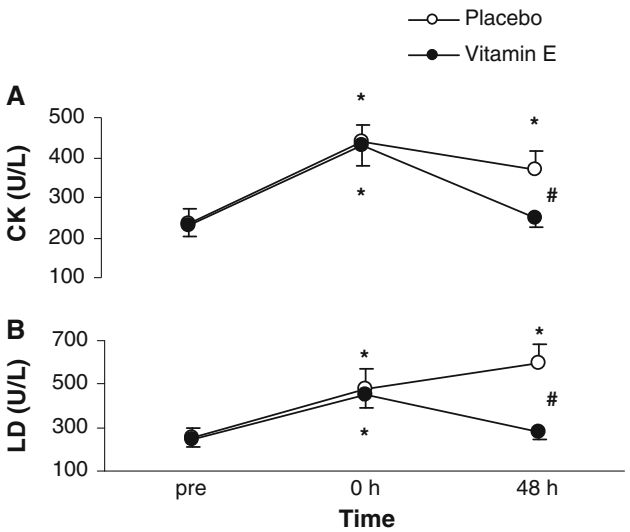

Fig. 1 Mean and SD ($n = 10$ per group) of plasma creatine kinase (**a**) and lactate dehydrogenase (**b**) in the placebo-treated and vitamin E-treated rats measured before, immediately after and 48 h after downhill running. *Significantly different from pre within the same group ($P < 0.001$); #significantly different between placebo and vitamin E at the same time point ($P < 0.001$)

Morphological alterations

When examined against the Pb–Sed rats, the Pb–Ex0 and Pb–Ex48 animals demonstrated moderate soleus muscle fibre damage. Most frequently observed histopathological changes included inflammatory neutrophil infiltration, fibre degeneration and fibre necrosis with macrophage invasion (Table 1). In the vitamin E-treated rats, exercise caused less pronounced and fewer morphological alterations of the soleus muscle (Fig. 2). Figure 2 depicts characteristic examples of each category of the morphological alterations presented in Table 1.

Functional alterations

A marginally nonsignificant supplementation-by-time interaction ($P = 0.062$) and significant main effects of supplementation ($P = 0.019$) and time ($P < 0.001$) in soleus muscle P_t were found (Fig. 3a). P_t was significantly lower in Pb–Ex0 ($P < 0.001$) and Pb–Ex48 ($P = 0.043$) compared to Pb–Sed, as well as significantly higher ($P < 0.001$) in Pb–Ex48 compared to Pb–Ex0. In the vitamin E groups, P_t decreased only immediately after exercise ($P = 0.003$) and was higher in VE–Ex0 compared to Pb–Ex0 ($P < 0.001$), suggesting an ameliorating effect of vitamin E on muscle injury immediately post-exercise. No significant supplementation-by-time interaction or main effects were found in TPT or $RT_{1/2}$ (Fig. 3b, c), therefore pairwise comparisons between the placebo and vitamin E groups were not performed. The effect sizes for Pt in the placebo groups at 0 and 48 h were −5.00 and −2.42, respectively, whereas the corresponding effect sizes in the vitamin E groups were −1.60 and −0.95.

Neither significant supplementation-by-time interaction nor significant main effects of supplementation ($P > 0.05$) in soleus muscle unfused tetanic force or P_o were found, therefore pairwise comparisons between the placebo and vitamin E groups were not performed. With respect to 40-Hz, unfused tetanic force, a significant main effect of time ($P < 0.001$) was observed (Fig. 3d). Unfused tetanic

Table 1 Main light microscopy morphological findings in rat soleus muscle following downhill running

Findings (% of fibres affected)	Pb–Ex0	VE–Ex0	Pb–Ex48		VE–Ex48	
	HE	HE	HE	T-blue	HE	T-blue
Edema, enlarged interstitial area	–	–	3.9 ± 0.70	1.9 ± 0.33	2.0 ± 0.56	–
Inflammatory neutrophil infiltration	1.8 ± 0.77	–	4.1 ± 0.95	4.0 ± 1.04	1.7 ± 0.76	–
Fibre degeneration, focal fibre necrosis (pale fibres)	3.7 ± 0.81	1.6 ± 0.59	1.8 ± 0.61	–	–	–
Fibre necrosis, macrophage invasion	1.6 ± 0.38	–	4.3 ± 1.10	1.7 ± 0.34	2.1 ± 0.67	2.0 ± 0.72

Mean and SD ($n = 6$ per group) expressed as percentage of fibres affected over the total number of fibres studied. A total of at least 300 myofibres from each rat were analysed: –, no damage; Pb–Ex0 and VE–Ex0, placebo-treated and vitamin E-treated, respectively, and studied immediately after exercise; Pb–Ex48 and VE–Ex48, placebo-treated and vitamin E-treated, respectively, and studied 48 h after exercise

HE hematoxylin and eosin stain, *T-blue* toluidine blue stain

force was significantly lower in Pb–Ex0 ($P < 0.001$) and Pb–Ex48 ($P = 0.049$) compared to Pb–Sed. When Pb–Ex48 was compared to Pb–Ex0, the 40-Hz force was marginally significantly higher ($P = 0.056$). In the vitamin E-supplemented groups, 40-Hz force significantly decreased only immediately after exercise ($P = 0.027$) and partially recovered 48 h post-exercise ($P > 0.05$). The effect sizes for the unfused tetanic force at 40 Hz in the placebo groups at 0 and 48 h were −3.10 and −1.56, respectively, whereas the corresponding effect sizes in the vitamin E groups were −1.34 and −1.03.

Regarding P_o, a significant main effect of time ($P < 0.001$) was also observed (Fig. 3e). Maximal tetanic force decreased significantly ($P < 0.001$) in Pb–Ex0 and marginally significantly ($P = 0.061$) in Pb–Ex48 compared to Pb–Sed. In the vitamin E groups, P_o did not change significantly immediately post- or 48 h post-exercise ($P > 0.05$). The effect sizes for P_o in the placebo groups at 0 and 48 h were −2.08 and −1.21, respectively, whereas the corresponding effect size in the vitamin E groups were −1.76 and −1.11.

Correlation analysis between alterations in histological, functional and biochemical measurements in soleus muscle at 48 h post-downhill running are presented in Table 2. Moderate correlations between histological changes and functional measures (P_t, P_o), as well as between histological changes and myofibre proteins in the circulation, (CK, LD) were observed.

Discussion

The higher plasma vitamin E levels of the supplemented rats in our study indicate that the administration protocol was effective in elevating vitamin E levels. In human studies, a typical method to boost vitamin E concentration in tissues is the ingestion of vitamin E at doses ranging from 300 to 1,200 mg daily (that is, 20–80 times the RDA) for a period of 1 week to more than 3 months (Bloomer 2007; Sacheck and Blumberg 2001). In animal studies, vitamin E supplementation is typically achieved through enriched diets or via injections (Van der Meulen et al. 1997; Warren et al. 1992). A daily i.p. injection of 100 mg/kg vitamin E for 7 days resulted in about a 3.5-fold increase in vitamin E content in the lungs (Kovacheva and Ribarov 1995), whereas the same regimen for 4 days decreased lipid peroxidation in the liver (Hidalgo et al. 1988). In addition, a 5-day vitamin E i.v. injection of a yet higher dose (0.2 ml of a 70% w/v vitamin E solution, equivalent to 400 mg/kg for a rat weighing 350 g), produced 2-to 3-fold increases in muscle vitamin E content (Van Der Meulen et al. 1997), which, according to the authors, were comparable to those achieved with 5 weeks of dietary supplementation with 10,000 IU vitamin E/kg diet (Van Der Meulen et al. 1997; Warren et al. 1992). To put it into perspective, the twofold increase in plasma vitamin E levels achieved in the supplemented rats in our study is consistent with the 2.5-fold higher serum vitamin E concentration in young men supplemented with 1,200 IU of vitamin E daily for 30 days (Beaton et al. 2002). Additionally, Hartman et al. (1995) have demonstrated that both plasma and muscle vitamin E content increased significantly after supplementing subjects with 1,200 mg of vitamin E daily for 14 days.

Our data indicate that the increased vitamin E levels caused by supplementation were not proven particularly effective in preventing exercise-induced injury in the soleus muscle. In the within groups comparisons, the vitamin E-treated animals preserved P_o immediately after and 48 h post-exercise. However, in the between groups comparisons at the respective time points, the decreased P_o values recorded in the placebo-treated rats were not significantly different from the P_o values observed in the vitamin E-treated rats. In this regard, vitamin E supplementation did not appear to be "physiologically" significant, as there was no difference between the placebo and vitamin E supplementation conditions in maximal tetanic

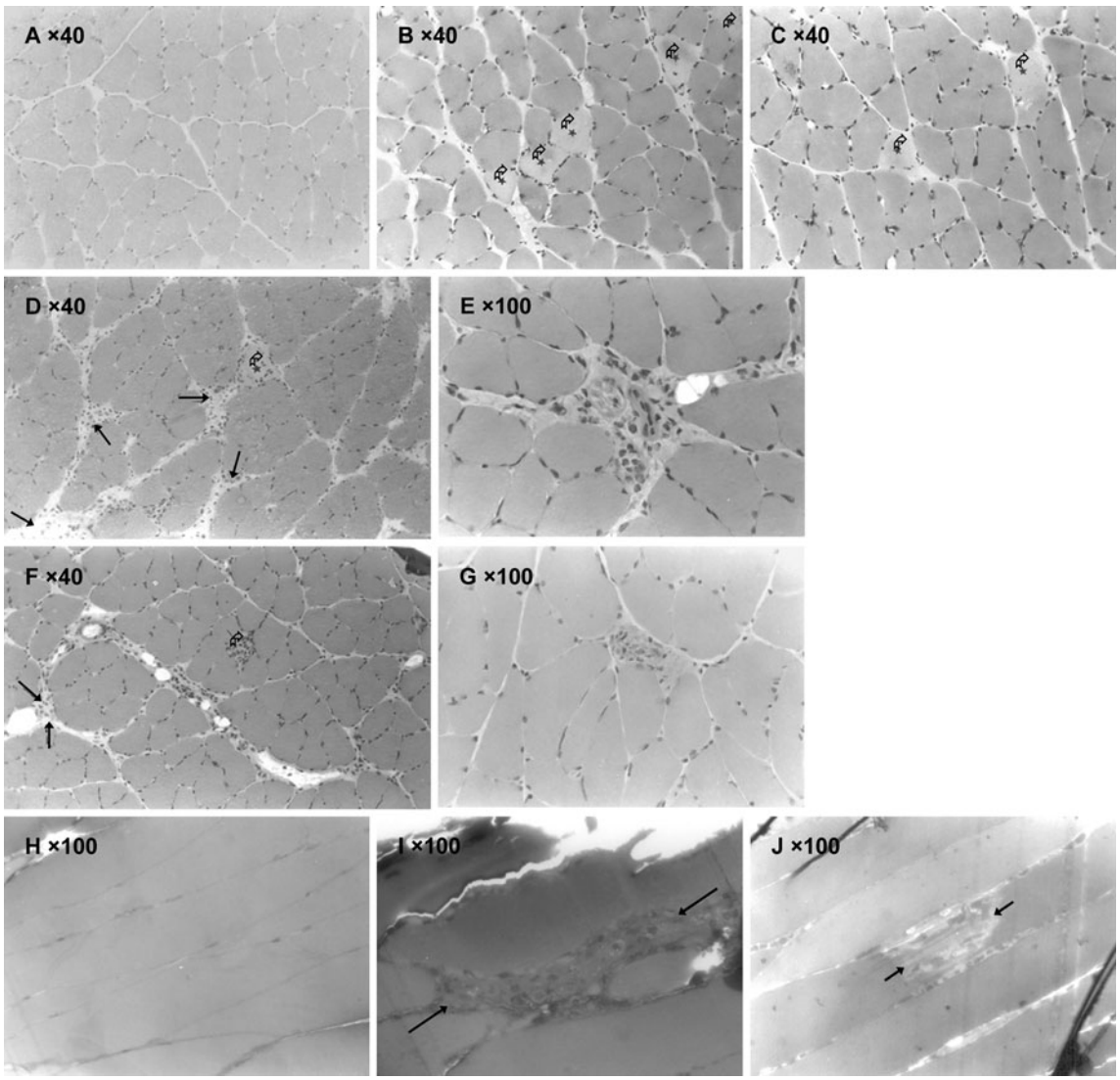

Fig. 2 Hematoxylin and eosin staining of frozen cross sections (**a**–**g**) and toluidine blue staining of longitudinal sections (**h**–**j**) of the soleus muscle from a sedentary placebo-treated rat (**a**, **h**), a placebo-treated rat examined immediately (**b**) or 48 h (**d**, **e**, **i**) after downhill running and a vitamin E-treated rat examined immediately (**c**) or 48 h (**f**, **g**, **j**) after downhill running. As opposed to the intact muscle fibres of **a**, **b** shows a greater number of pale (ghost) fibres (five) than that of **c** (two), indicative of greater degree of degeneration. **d** Extended edema, inflammatory neutrophil infiltration (four *arrows*) and necrotic fibre (*asterisk*) are shown. **e** Extended fibre necrosis and macrophage invasion are seen. **f** Moderate edema, moderate inflammatory neutrophil infiltration (two *arrows*) and necrotic fibre (*asterisk*) are present. **g** Focal fibre necrosis and macrophage invasion are observed. In toluidine blue-stained sections, contrary to the intact muscle fibres in **h**, extended fibre necrosis with macrophage invasion (*long arrows*) is visible in **i** of the placebo-treated group 48 h post-exercise, whereas focal fibre necrosis with some macrophage invasion (*short arrows*) is seen in **j** of the vitamin E-treated group 48 h post-exercise

force, a reliable measure of muscle function. In addition, vitamin E treatment did not prevent the elevation in plasma CK and LD immediately post-exercise.

However, there are secondary findings suggesting yet another positive role of vitamin E supplementation in attenuating exercise-induced injury markers. As compared to the placebo-treated rats, the vitamin E-treated animals had significantly higher soleus muscle P_t immediately post-exercise, significantly reduced plasma CK and plasma LD 48 h post-exercise and less pronounced morphological alterations in muscle immediately and 48 h post-exercise. Furthermore, in the within groups comparisons, the placebo-treated animals had significant alterations in all injury markers measured. In contrast, vitamin E-treated rats had decreased soleus muscle P_t and unfused tetanic force as well as increased plasma CK and LD activity immediately post-exercise, but these markers returned to the baseline values at 48 h post-exercise.

These findings are in partial agreement with the data of the two relevant animal studies found in the literature (Van

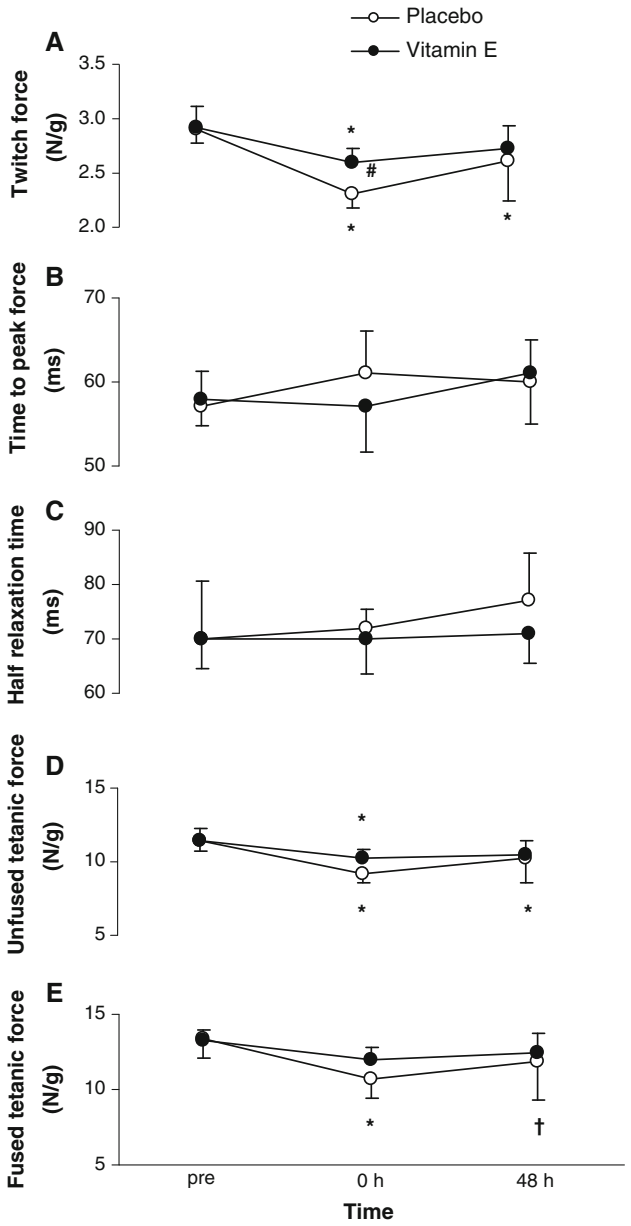

Fig. 3 Mean and SD ($n = 10$ per group) of in situ isometric single-twitch properties (**a**–**c**), unfused tetanic force at 40 Hz (**d**) and isometric maximal (fused) tetanic force (P_o) at 80 Hz (**e**) of the soleus muscle in the placebo-treated and vitamin E-treated rats recorded before, immediately after and 48 h after downhill running. *Significantly ($P < 0.05$) or †borderline significantly ($P = 0.061$) different from pre within the same group; #significantly ($P < 0.05$) different between placebo and vitamin E at the same time point

der Meulen et al. 1997; Warren et al. 1992). The latter employed a downhill running protocol of similar inclination to that used in our study (i.e. -17^{o} vs. -16^{o}) but of higher intensity (25 vs. 16 m/min) and longer duration (150 vs. 90 min). The authors found that a 3- to 4-fold rise in vitamin E in the soleus muscle was accompanied by decreased susceptibility of the muscle homogenates to oxidative stress. However, there was no protection against the reduction in maximal tetanic force, muscle fibre damage or CK elevation immediately and 48 h post-exercise. Likewise, Van der Meulen et al. (1997), utilizing a well-defined and highly reproducible muscle injury model (225 lengthening contractions applied to rat extensor digitorum longus muscle in situ), reported that a threefold rise in muscle vitamin E prevented the fourfold and twofold increase in serum CK activity observed 3 and 3 days, respectively, after the end of the lengthening contraction protocol in control. Nevertheless, there was no protection against maximal tetanic force reduction or muscle fibre damage measured 3 days after exercise.

This inconsistency in the effectiveness of vitamin E may be partly explained by the greater magnitude of muscle damage reported in the two aforementioned studies compared to our study. We found that, in the placebo-treated exercised rats, P_o decreased by about 20 and 11.5% immediately after and 48 h after exercise, respectively, whereas the decrease was less than 10% at both time points in the vitamin E-treated exercised rats. In the study of Warren et al. (1992), P_o was decreased by 20% both immediately after and 48 h after exercise in both the placebo treated and vitamin E-treated exercised groups. The P_o effects sizes were higher in our study compared to that of Warren et al. (1992). In our study, the effect sizes for the P_o in the placebo groups at 0 and 48 h were -2.08 and -1.21, respectively, whereas the corresponding effect sizes in the aforementioned study were -1.11 and -1.10, as estimated from the figures. Likewise, the effect sizes for the P_o in the vitamin E groups at 0 and 48 h were -1.76 and -1.11, respectively, whereas the corresponding effect sizes in the aforementioned study were -0.92 and -0.81.

In the study of Van der Meulen et al. (1997), the P_o deficit was about 64% at both 30 min and 3 days post-exercise, and it was not attenuated by vitamin E supplementation. With regard to muscle histology, the fibre damage in our study was much less severe compared to the figures of 30 and 64% damaged fibres reported by Warren et al. (1992) and Van der Meulen et al. (1997), respectively. Furthermore, in the present study, the post-exercise increase in plasma CK concentration was less than 100%, whereas the respective increases in the aforementioned studies were about 200 and 400%, respectively. The effect sizes for CK in the placebo groups at 0 and 48 h were 5.59 and 3.67, respectively (compared to 4.7 and 0.3 in the study of Warren et al. 1992, as estimated from the figures). Likewise, the corresponding effect sizes for CK in the vitamin E groups were 6.99 and 3.67 (compared to 5.7 and 1.7 in the aforementioned study).

It appears that the time courses of progression of the indices commonly used to detect muscle injury do not coincide. Whereas muscle function/force is impaired

Table 2 Correlation analysis between changes in histological, functional and biochemical measurements in soleus muscle at 48 h post-downhill running

	P_t	TTP	$RT_{1/2}$	40-Hz tension	P_o	CK	LD
Edema, enlarged interstitial area	–0.582*	–0.286	0.186	–0.474	–0.579*	0.642*	0.716*
Inflammatory neutrophil infiltration	–0.450	–0.408	0.179	–0.460	–0.598*	0.696*	0.777*
Fibre necrosis, macrophage invasion	–0.562	–0.308	0.242	–0.359	–0.608*	0.742*	0.647*

Spearman's rank correlation coefficient (total $n = 12$ rats). To increase the statistical power, values of the Pb–Ex48 ($n = 6$) and VE–Ex48 ($n = 6$) groups were pooled together. Histological alterations were based on hematoxylin and eosin stain

P_t single-twitch tension, *TPT* time-to-peak tension, $RT_{1/2}$ single-twitch half-relaxation time, P_o maximal tetanic force, *CK* creatine kinase, *LD* lactate dehydrogenase

* $P < 0.05$

immediately after the injury protocol and recovers slowly over the next days or weeks, other muscle injury markers, including the release of myofibre proteins and histopathology findings, are not evident over the entire time course of muscle injury, and, when evident, may not correlate with the functional decrements. In humans, peak blood CK levels occur no earlier than 1 day after exercise. In animals, the manifestation of myofibrillar proteins in blood appears to be biphasic. An immediate increase is associated with the initial mechanical injury and a second peak, 2–4 days later, is related to the infiltration of phagocytic cells (Warren et al. 1999).

Our findings regarding CK and LD activities suggest that the protective effect of vitamin E was time dependent. The increased blood CK and LD activities immediately after exercise, possibly reflecting sarcolemma disruption due to the initial mechanical insult of eccentric exercise (Armstrong 1990; Clarkson and Sayers 1999), do not support the efficacy of vitamin E as a membrane stabilizer. However, the attenuation of CK and LD activities in the vitamin E-treated rats 48 h post-exercise (a time point corresponding to the phagocytic stage characterized by oxidative damage, as mentioned above), supports an antioxidant role of vitamin E. Myofibre proteins in the circulation may provide a gross indication of muscle injury and particularly sarcolemma integrity, but do not necessarily help to estimate the extent of injury (Fridén and Lieber 2001), as blood protein levels depend on not only the release of proteins into the circulation but also on their clearance (Warren et al. 1999). In addition, changes in blood levels of myofibre proteins correlate poorly with other muscle injury markers, including histological abnormalities and functional losses (Fridén and Lieber 2001; Warren et al. 1999).

With regard to the histological alterations observed in the present study, although the vitamin E-supplemented rats exhibited less damage to muscle fibre morphology, the injury was not prevented. This finding is generally in line with those of the two studies in animals mentioned above (Van der Meulen et al. 1997; Warren et al. 1992) and of a study in humans (Beaton et al. 2002) showing that vitamin E supplementation did not prevent the damage to the histological appearance of the myofibres. Despite the apparent sampling and tissue analysis limitations, histology has long been incorrectly considered as the reference injury criterion. However, histological analysis fails to evaluate precisely the extent of injury (Warren et al. 1992). What is more, it has been demonstrated that neither the time course nor the magnitude of histological abnormalities corresponds with the alterations in muscle function (Warren et al. 1999).

It has been proposed that muscle function, particularly the measurement of the electrically induced isometric force in animal studies and maximal voluntary contraction torque in human studies, is the best index of muscle injury induced by eccentric exercise (Warren et al. 1999). An interesting finding of our study was the greater soleus muscle P_t in the vitamin E-treated animals post-exercise, as compared to their placebo-treated counterparts, despite the nonsignificant difference in P_o. Unlike P_o, which results from a train of stimuli causing prolonged and maximal Ca^{2+} release from the sarcoplasmic reticulum (SR), P_t results from a single opening of the SR Ca^{2+} channels causing a transient and limited Ca^{2+} release (Eusebi et al. 1980). In this regard, P_t may be a more sensitive index of the normal Ca^{2+} release process compared to tetanic force and, thus, more likely to detect impairment in Ca^{2+} transients in injured myofibres at the microtrauma level. In contrast, the high frequency of stimuli applied to generate P_o may compensate for a potential impairment in Ca^{2+} transients that would normally be manifested if the stimuli were of lower frequency. In support of a partial protective effect of vitamin E against muscle injury, $RT_{1/2}$ tended to be lower 48 h post-exercise in the vitamin E-treated animals, which may reflect an increased rate of Ca^{2+} reuptake by the SR. A depression in the rate of Ca^{2+} uptake by the

SR has been primarily attributed to a reduced Ca^{2+}-stimulated ATPase activity (Yasuda et al. 1999) and to the mechanical injury of the SR induced by eccentric contractions (Yasuda et al. 1997).

Our findings regarding the poor effects of vitamin E on the reduction in maximal tetanic force are generally in line with those of previous animal (Van der Meulen et al. 1997; Warren et al. 1992) and human studies (Beaton et al. 2002; Bloomer et al. 2004; Jakeman and Maxwell 1993), which reported that vitamin E treatment did not prevent muscle force deficit caused by eccentric exercise. However, Shafat et al. (2004) recently demonstrated an attenuation of force deficit after supplementation with a combination of vitamins C and E.

When combined, our muscle functional and morphological findings support the prevailing mechanisms put forward to explain the decrease in muscle force induced by eccentric contractions. These are excitation–contraction (E–C) coupling failure (Ingalls et al. 2004; Takekura et al. 2001; Warren et al. 2001), structural damage of the myofibrils resulting in disorganization of the contractile machinery (Ingalls et al. 1998) and lower myofibrillar Ca^{2+} sensitivity (Lee et al. 1991). With regard to the protective effect of vitamin E supplementation on muscle single-twitch force found immediately after exercise, it is postulated that this effect may be mainly related to the E–C coupling process rather than to contractile machinery damage, yet the exact mechanism through which vitamin E exerts its action is still unknown. It has been suggested that the E–C coupling failure could explain at least 75% of P_o decrease immediately after eccentric contraction and it was still responsible for at least 57% of P_o reduction in 5 days (Ingalls et al. 1998).

It is important to note that the magnitude and time course of the changes in muscle injury parameters are different following eccentric exercise (Fridén and Lieber 2001; Warren et al. 1999). For example, dissociation between histological changes and muscle function alterations was reported (Warren et al. 1999). The present study found only moderate correlations between histological changes and other injury markers. Considering the fact that recovery of muscle function after exercise is the most important aspect for prophylactic or therapeutic interventions, we propose that markers of muscle function such as P_o, are more important than other injury markers assessed in the present study when determining the effects of vitamin E supplementation on muscle injury.

A limitation of the present study was that vitamin E concentration was not determined in the soleus muscle for technical reasons. Had we measured muscle vitamin E content, a more straightforward relationship between muscle vitamin E concentration and the potential attenuation of muscle injury might have ensued. In addition, the experimental design of this study did not allow the investigation of the role of vitamin E in the progression of muscle injury and the subsequent recovery throughout the entire time course of muscle degeneration and regeneration as only two sampling time points, namely immediately after and 48 h after exercise, were examined.

Another limitation of the present study was that we did not determine oxidative stress biomarkers which could have provided an estimate of the contribution of reactive oxygen or nitrogen species to muscle injury induced by eccentric exercise and of whether vitamin E alleviated oxidative stress. It was assumed that the increased vitamin E concentration within the supplemented groups would also provide increased antioxidant protection to the muscle cell membranes. As demonstrated by You et al. (2005), a significant increase in protein carbonyls, but not in malondialdehyde or oxidized glutathione, occurred in rat plasma, vastus intermedius muscle and soleus muscle after a downhill running protocol identical to that used in the present study. Supplementation with a combination of vitamins C and E decreased protein carbonyls in both muscles. These data were in line with the decreased susceptibility of the soleus muscle homogenates to oxidative stress in vitamin E-treated rats after downhill running (Warren et al. 1992). The report of reduced muscle conjugated dienes and urinary thiobarbituric acid-reactive substances in vitamin E-treated subjects following downhill running (Meydani et al. 1993) provide further evidence for the role of vitamin E in reducing eccentric exercise-induced oxidative stress.

In conclusion, the results of the present study together with the findings of the previous studies (Van der Meulen et al. 1997; Warren et al. 1992) suggest that a short-term vitamin E supplementation is not effective in preventing or attenuating decreases in muscle function after eccentric exercise with physiological significance. However, the effects of the vitamin E supplementation on muscle morphological changes and myofibre proteins in the circulation shown in the present study can be considered to be a positive sign. Therefore, our data suggest a more positive role of vitamin E than that suggested by the two previous studies of similar design to ours. This difference may be explained by differences in the characteristics of the eccentric exercise protocols employed to cause muscle fibre damage, while it is unclear whether vitamin E dosage played any role. Nevertheless, as optimal cellular function is directly related to physical capacity, any protection against exercise-induced muscle injury by vitamin E may prove beneficial in maintaining muscle function and preserving athletic performance. Thus, vitamin E supplementation at the doses and duration administered in the present study was not proven particularly effective against eccentric exercise-induced muscle damage. Future studies could

focus on the relationship between the extent of muscle injury and from a hormesis point of view the most effective dosage of vitamin E required to prevent it.

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Conflict of interest The authors declare no conflict of interest.

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