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Title: Oxidative Stress, Inflammation and Recovery of Muscle Function after Damaging Exercise: Effect of 6-weeks Mixed Antioxidant Supplementation.

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ABSTRACT

There is no consensus regarding the effects of mixed antioxidant vitamin C and/or vitamin E supplementation on oxidative stress responses to exercise and restoration of muscle function. Thirty-eight men were randomly assigned to receive either placebo group (n=18) or mixed antioxidant (primarily vitamin C & E) supplements (n=20) in a double-blind manner. After 6-weeks, participants performed 90 minutes of intermittent shuttle-running. Peak isometric torque of the knee flexors/extensors and range of motion at this joint were determined before and after exercise, with recovery of these variables tracked for up to 168 h post-exercise. Antioxidant supplementation elevated pre-exercise plasma vitamin C (93±8 $\mu mol \cdot l^{-1}$) and vitamin E (11±3 $\mu mol \cdot l^{-1}$) concentrations relative to baseline ($P<0.001$) and the placebo group ($P \leq 0.02$). Exercise reduced peak isometric torque (i.e. 9-19% relative to baseline; $P \leq 0.001$), which persisted for the first 48 hours of recovery with no difference between treatment groups. In contrast, changes in the urine concentration of F_2-isoprostanes responded differently to each treatment ($P=0.04$), with a tendency for higher concentrations after 48 h of recovery in the supplemented group (6.2±6.1 $ng \cdot ml^{-1}$ *versus* 3.7±3.4 $ng \cdot ml^{-1}$). Vitamin C & E supplementation also affected serum cortisol concentrations, with an attenuated increase from baseline to the peak values reached after 1 h of recovery compared with the placebo group ($P=0.02$) and serum interleukin-6 concentrations were higher after 1 h of recovery in the antioxidant group (11.3±3.4 $pg \cdot ml^{-1}$) than the placebo group (6.2±3.8 $pg \cdot ml^{-1}$; $P=0.05$). Combined vitamin C & E supplementation did not reduce markers of oxidative stress or inflammation nor did it facilitate recovery of muscle function after exercise-induced muscle damage.

Key Words: ASCORBIC ACID, RRR-α-TOCOPHEROL, CYTOKINES, MUSCLE DAMAGE

INTRODUCTION

Exercise places a degree of mechanical and metabolic stress on the body, which jointly contribute to a commonly-experienced sub-clinical pathological response involving oxidative stress and subsequent inflammation (Pyne 1994). On one hand, this response plays a key role in the repair and regeneration of tissue as part of a long-term adaptive process, such that any interventions designed to acutely attenuate these responses have impaired training adaptations in some (Gomez-Cabrera et al. 2008) but not all (Yfanti et al. 2010) studies. However, when coupled with an awareness of the distinction between physiological responses to chronic physical training and pathophysiological responses to an acute excessive overload of exercise (Brigelius-Flohe 2009), this view can be balanced against the more immediate implications of exercise-induced muscle damage in terms of transient sensitisation of afferent nerve endings, compromised integrity of myofibrillar contractile proteins and reduced insulin action (Friden and Lieber 1992; Kirwan et al. 1992; Smith 1991). The resultant pain and impairment of muscular function and muscle glycogen resynthesis (Costill et al. 1990) after initial tissue injury thus have relevance to, for example: a relatively sedentary individual at the outset of an exercise program; a habitual exerciser planning a single novel activity beyond their usual regimen; and/or an athlete returning to sport after injury. These populations would benefit from interventions to alleviate such symptoms in the short-term (Howatson and van Someren 2008), either to facilitate adherence to an exercise program or to offset decrements in performance.

Antioxidant vitamins are prime examples of such interventions. Our previous work provided evidence for a modest beneficial effect of 2-weeks vitamin C supplementation in relation to changes in muscle function/soreness and certain aspects of oxidative stress (i.e. lipid peroxidation) and inflammation (i.e. cytokine response) after intermittent exercise (Thompson et al. 2001). This benefit of vitamin C alone is consistent with other studies that have observed improved recovery of maximum voluntary contraction (Jakeman and Maxwell 1993) and

evidence of reduced lipid peroxidation (Alessio et al. 1997) following exercise-induced muscle damage. Interestingly, the latter of these studies also observed a benefit of 21-days supplementation with vitamin C even relative to vitamin E (Jakeman and Maxwell 1993). This result is of relevance given that others have reported independent effects of vitamin E in terms of reducing post-exercise markers of lipid peroxidation (i.e. urinary exretion of thiobarbituric acid adducts; Meydani et al. 1993) and secretion of cytokines involved in the inflammatory process, such as interleukin-6 and -1β (Cannon et al. 1991). In contrast, when the activity of plasma creatine kinase has been used as an indirect measure of exercise-induced muscle damage, vitamin E supplementation alone has not reduced the efflux of this enzyme into the circulation (Cannon et al. 1990; Warren et al. 1992).

In addition to the effects of ingesting single antioxidants, more recent studies have explored the potential benefits of mixed antioxidants that are popular commercial products and may offer better synergism between various interrelated antioxidant networks *in vivo* (Powers et al. 2010). Most commonly, vitamin C (ascorbic acid) and vitamin E (α-tocopherol) have been co-ingested (Bloomer et al. 2007; Bloomer et al. 2006; Dawson et al. 2002; Fischer et al. 2004; Howatson et al. 2009; Mastaloudis et al. 2004; Mastaloudis et al. 2006; Petersen et al. 2001; Rokitzki et al. 1994), although others have examined combined vitamin C and E supplements that also include smaller quantities of vitamin A (α-/β-carotene) precursors (Kanter et al. 1993; Machefer et al. 2007; Schroder et al. 2000) or selenium (Goldfarb et al. 2005), among other ingredients (i.e. lutein, zinc and magnesium; Teixeira et al. 2009). These supplements have been evaluated using different types of exercise and, while some studies have reported reduced evidence of oxidative stress associated with vitamin C & E supplementation (Bloomer et al. 2006; Fischer et al. 2004; Goldfarb et al. 2005; Machefer et al. 2007; Mastaloudis et al. 2004; Schroder et al. 2000), others report no difference relative to placebo supplementation (Bloomer et al. 2007; Bloomer et al. 2006; Dawson et al. 2002; Kanter et al. 1993; Rokitzki et al. 1994; Teixeira et al. 2009). There appears to be more consistent results in relation to inflammatory

responses; the majority of studies report no effect of vitamin C & E supplementation on systemic cytokine responses following exercise (Bloomer et al. 2007; Mastaloudis et al. 2004; Petersen et al. 2001; Teixeira et al. 2009), although others have shown reduced concentrations of interleukin-6 and interleukin-1ra (Fischer et al. 2004). Interestingly, that study also elegantly illustrated that this effect on interleukin-6 was attributable primarily to a net reduction in release from previously exercised skeletal muscle (Fischer et al. 2004).

Notwithstanding the equivocal evidence regarding the effects of vitamin C & E supplementation on oxidative stress and inflammation, direct histological examination of muscle tissue via electron microscopy has not revealed any less ultrastructural disruption after exercise when supplemented with these vitamins (Dawson et al. 2002). This finding is entirely consistent with every study to have reported blood-borne biomarkers (e.g. creatine kinase, myoglobin and lactate dehydgrogenase) as proxy measures of muscle damage (Bloomer et al. 2007; Dawson et al. 2002; Machefer et al. 2007; Mastaloudis et al. 2006; Petersen et al. 2001; Rokitzki et al. 1994; Teixeira et al. 2009), none of which has reported significant protective effects of combined vitamin C & E supplementation. From this perspective, it is noteworthy that such biomarkers do not correlate well either with the magnitude of tissue injury (Komulainen et al. 1995) or impairment of muscle function (Margaritis et al. 1999), the latter of which is believed to represent the most valid indirect predictor of the degree of muscle damage sustained (Clarkson and Hubal 2002). Combined with the fact that recovery of muscle function after exercise is also of clear practical value, it is interesting that only two studies have monitored the response of this variable to combined vitamin C & E intervention over the days following exercise (Bloomer et al. 2007; Mastaloudis et al. 2006). Of these, neither reported any benefit of specific vitamin C & E supplements in relation to either muscle-specific resistance exercise (Bloomer et al. 2007) or ultra-endurance exercise (Mastaloudis et al. 2006). However, these exercise models may not reflect the muscle damage typically experienced across a range of muscle groups during more common activities such as basketball, soccer or tennis involving high metabolic demand in

104 combination with repeated eccentric or unaccustomed muscle actions (i.e. high-intensity
105 intermittent shuttle-running; Thompson et al. 1999).
106
107 In summary, the potential for combined vitamin C & E supplementation to impact upon
108 oxidative stress, inflammation and systemic indices of muscle damage after exercise remains
109 uncertain and very little information is available regarding the recovery of muscle function. The
110 aim of the present study was therefore to examine the influence of 6-weeks of mixed antioxidant
111 (primarily vitamin C & E) supplementation on oxidative stress, inflammation and the functional
112 recovery of skeletal muscles after prolonged intermittent shuttle-running. Based upon reports
113 that all these outcomes can be affected when vitamin C or E are supplemented individually
114 (Alessio et al. 1997; Cannon et al. 1991; Jakeman and Maxwell 1993; Meydani et al. 1993;
115 Thompson et al. 2001), it is hypothesised that mixed antioxidant vitamin C & E supplementation
116 will attenuate these responses and that, given the inhibitory effect of these processes on
117 excitation-contraction coupling (Brown et al. 1996), this may translate into improved recovery of
118 muscle function.
119

MATERIALS AND METHODS

Approach to the Research Question

The primary purpose of this study was to examine whether vitamin C & E supplementation presents any benefit in terms of facilitating functional recovery following exercise-induced muscle damage. Given the inherent inter-individual variance associated with many measures of exercise-induced muscle damage, an ideal approach is to adopt a repeated-measures cross-over design when assessing the efficacy of nutritional interventions (Betts et al. 2009) and one study has employed this approach in relation to combined vitamin C & E supplements (Dawson et al. 2002). However, in view of the slow intracellular turnover of vitamin E in many tissues (i.e. adipose tissue, adrenal glands, liver and skeletal muscle fibres; Packer 1992), it was decided that an independent-measures 2-group design would be most appropriate for this study but with a larger sample size (n = 18 & 20) than has previously been used in thus type of study (typically n = 10) in order to account for individual differences.

This relatively large cohort of participants were randomly assigned to receive either a placebo supplement or a mixed antioxidant (primarily vitamin C & E) supplement for a period of 6 weeks prior to completing a high-intensity intermittent shuttle-running protocol. The use of this protocol both increases ecological validity with specific reference to participation in team-sports and also has the potential to induce substantial muscle damage on a whole-body level through the combination of eccentrically-biased muscle actions (e.g. decelerations from repeated sprints and frequent changes of direction) and high metabolic demand (Thompson et al. 1999). Furthermore, as has recently been advocated (Powers et al. 2010), the antioxidant mixture provided in this study was a cocktail including not only vitamins C and E but also lesser quantities of other nutrients (detailed under ‘Supplementation’) that act synergistically with more potent or direct antioxidants to protect against oxidative damage. Obviously, should any

differences between treatments occur, a subsequent investigation would be required to isolate the mechanisms through which specific antioxidants operate.

We employed multiple outcome measures to provide a more valid overall interpretation than would be possible using any one in isolation. In particular, serial assessments of muscle function for up to a week following exercise represents a central novel element of this study, while also affording high levels of both internal and external validity (Clarkson and Hubal 2002). Furthermore, the measures of oxidative stress most commonly used in this area (e.g. thiobarbituric acid adducts/malondialdehyde) are not specific to oxidative processes (Halliwell and Chirico 1993), whereas urinary F_2-isoprostanes are more reliable biomarkers of lipid peroxidation *in vivo* (Liu et al. 2009; Powers et al. 2010) and so were used in the present study. For completeness and to facilitate comparisons with existing research on this topic, indirect markers of skeletal muscle damage and inflammation were also determined in venous blood samples collected over the hours and days following exercise-induced muscle damage.

Participants

Thirty-eight healthy young men volunteered to take part in this study and their characteristics were as follows: age 22 ± 1 yr, body mass (BM) 77.0 ± 8.9 kg (beam balance scales, Avery Ltd), height 1.78 ± 0.06 m (Stadiometer, Holtain Ltd), Body Mass Index 24 ± 2 $kg \cdot m^{-2}$, Σ triceps, biceps, subscapular and suprailiac skin-folds 34 ± 9 mm (callipers, Salter; ACSM 1995) and $\dot{V}O_{2\,max}$ 55 ± 5 $mL \cdot kg^{-1} \cdot min^{-1}$ (means ± SD). All participants were non-smokers and habitually active in a variety of sports but were unfamiliar with the specific exercise protocol involved in this investigation. Once fully briefed regarding the nature of the study both verbally and in writing, each participant provided written informed consent in keeping with the requirements of the Loughborough University Ethical Advisory Committee who approved this study.

171 **Experimental Design**

172 This experiment adopted an independent-measures 2-group design whereby each
173 participant was randomly allocated in a double-blind manner either to a group receiving placebo
174 supplements (n=18) or a group receiving mixed antioxidant vitamin C & E supplements (n=20).
175 Over a period of 6 weeks, these supplements were ingested as part of participants' habitual diets
176 (assessed via 5-day food records and dietary analysis software: COMP-EAT 4.0, Nutrition
177 Systems), which did not differ between groups (Placebo: 13381 ± 3940 $kJ \cdot d^{-1}$, 54 ± 7%
178 carbohydrate, 30 ± 6% fat, 16 ± 4% protein, vitamin C 123 ± 79 $mg \cdot d^{-1}$, vitamin E 7.7 ± 2.9
179 $mg \cdot d^{-1}$ *versus* Antioxidant: 13415 ± 2935 $kJ \cdot d^{-1}$, 53 ± 5% carbohydrate, 31 ± 6% fat, 16 ± 3%
180 protein, vitamin C 120 ± 30 $mg \cdot d^{-1}$, vitamin E 8.7 ± 4.6 $mg \cdot d^{-1}$; mean ± SD). This
181 supplementation regimen was continued for two days after an exercise protocol that required
182 participants to perform approximately 90 min of intermittent high-intensity shuttle-running (a
183 schematic of the study timeline is available via the EJAP online as Electronic Supplementary
184 Material).
185
186 **Preliminary Measurements and Familiarisation**

187 The exercise intensities employed during the study were calculated relative to each
188 participant's maximal oxygen uptake, determined prior to supplementation using a progressive
189 shuttle-running protocol to exhaustion as previously described (Nicholas et al. 2000). During the
190 first month of supplementation participants made repeated (≥3) visits to the laboratory until they
191 were fully familiar with the CYBEX NORM^{TM} isokinetic dynamometer (model 770), thus
192 establishing sufficiently reliable baseline measurements of peak isometric torque in knee flexors
193 (co-efficient of variation = 6 ± 4%) and extensors (co-efficient of variation = 8 ± 5%) of each
194 participant's non-dominant limb. For this test, participants completed a 5-min warm-up using a
195 cycle ergometer (Monark 863e) at 100 W, before being individually fitted and secured into the
196 dynamometer with knee movement restricted to the saggital plane and the axis of

197 extension/flexion through the femoral condyles. The maximal range of motion about the knee
198 was then ascertained, followed by a further isokinetic warm-up involving five flexions and
199 extensions at 1.05 rad·s^{-1}. Peak isometric torque was then determined as the maximal rotational
200 force voluntary achieved across 2 sustained efforts, each lasting 5 s and separated by a rest
201 interval of 60 s. These measurements were made while the participant was in an upright seated
202 position (hip angle 105º relative to full extension) at knee angles of 60º and 20º relative to full
203 extension for assessment of the knee extensors and flexors, respectively, because these joint
204 angles are optimal for peak isometric torque production in each muscle group (Ng et al. 1994;
205 Onishi et al. 2002). Venous blood and urine samples were also obtained at baseline prior to
206 supplementation.
207
208 **Experimental Protocol**
209 On the fortieth day of supplementation, participants arrived in the laboratory in the
210 morning following a 10 h overnight fast and having refrained from strenuous physical activity
211 for at least 48 h. Participants provided pre-exercise ratings of their perceived degree of muscle
212 soreness using a 1-10 scale with anchor terms ranging from 1 being "Not Sore" to 10 being
213 "Very Very Sore", as has been employed in our previous study (Thompson et al. 2001; this scale
214 is available via the EJAP online as Electronic Supplementary Material). An 8 mL resting blood
215 sample was then obtained via venepuncture from an antecubital vein and a pre-exercise urine
216 sample was also collected. A pre-exercise assessment of muscle function was then carried out
217 exactly as described in relation to 'Preliminary Measurements and Familiarisation'. Participants
218 then drank a volume of plain water (5 mL·kg BM^{-1}) before the exercise protocol was initiated.
219 The exercise protocol involved ~90 min of intermittent shuttle-running at intensities relative to
220 each participant's $\dot{V}O_{2max}$ (Loughborough Intermittent Shuttle Test; Nicholas et al. 2000). The
221 90 min was divided equally into 6 blocks each separated by a 3 min rest period during which
222 further volumes (2 mL·kg BM^{-1}) of plain water were ingested. Heart-rate (Sports Tester PE3000,

Polar, Finland) and ratings of perceived exertion (Borg 1973) were measured at regular intervals during exercise. Ambient temperature and humidity were closely matched between treatments (Placebo: 16 ± 1ºC, 54 ± 6% *versus* Antioxidant: 16 ± 1ºC, 55 ± 6%).

Immediately after exercise, a venous blood sample was drawn by venepuncture followed by an assessment of muscle function. Thereafter, participants rested in the laboratory for 1 h before a further follow-up blood sample was obtained by venepuncture. Supplements ingested on test days were taken only after this final blood sample had been consumed, coincident with the participants' next meal. Approximately (± 2 h) 24 and 48 h following the cessation of exercise, participants returned to the laboratory to provide venous blood and urine samples, along with measures of muscle function. Ratings of perceived muscle soreness were again obtained from participants at these time points and at 12 h intervals in between visits. A sub-population of the total cohort (n = 11) also provided additional follow-up measurements of muscle function after 96 and 168 h of recovery.

Supplementation

The antioxidant and placebo supplements were randomly assigned in a double-blind manner in the form of identical capsules, ingested twice daily with water following meals. Each antioxidant capsule provided 400 mg vitamin C (ascorbic acid), 268 mg vitamin E (RRR-α-tocopherol), 2 mg vitamin B6 (pyridoxine hydrochloride), 200 μg vitamin B9 (folic acid), 5 μg zinc sulphate monohydrate and 1 μg Vitamin B12 (cyanocobalamin), while placebo capsules contained only lactose (RP Scherer Limited, UK). When considered within the context of participants' habitual diets, antioxidant supplementation therefore resulted in approximately an 8-fold increase in daily vitamin C intake and approximately a 160-fold increase in daily vitamin E intake. This therefore represents >1000% and ~3600% of the recommended daily allowance for vitamin C and E, respectively, but well within the upper limit that poses risk of serious

adverse effects for almost all individuals in the general population (Hathcock et al. 2005). This dose was chosen as it is reflective of that found in commercially available supplements and is also typical of that used in previous studies where effects on oxidative stress and inflammation have been detected (Bloomer et al. 2006; Fischer et al. 2004; Goldfarb et al. 2005; Machefer et al. 2007; Mastaloudis et al. 2004; Schroder et al. 2000).

Sampling and Analyses

From each 8 mL blood sample, 4 mL was dispensed into a non-anticoagulant tube where it was left to clot for 45 min at room temperature and then centrifuged at 4000 *g* for 15 min at 4 ºC. The serum fraction was then stored at -80 ºC pending analyses at 37ºC using commercially available enzymatic colorimetric assays for myoglobin, creatine kinase and uric acid (Randox, UK) and an automated spectrophotometric analyser (COBAS-Mira plus, Roche) and for cortisol via radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, UK) and an automated gamma counter (Cobra II, Packard Instruments Company Inc, US). Where sufficient serum was available for each participant, concentrations of interleukin-6 (R&D Systems Inc. UK), interleukin-1 receptor antagonist (R&D Systems Inc. UK), C-reactive protein (DSL, UK), heat shock protein (HSP)70 (Stressgen Biotechnologies Inc. USA) and tumor necrosis factor (TNF)-α (R&D Systems Inc. UK) were determined using commercially available Enzyme-Linked ImmunoSorbent Assays (ELISA) with a spectrophotometric plate reader (Dynex Technologies Inc. USA). The remaining 4 mL of whole-blood was transferred into a tube containing the anticoagulant ethylenediaminetetraacetic acid (EDTA), from which triplicate 50 µl and 20 µl samples were taken for the respective manual determination of haematocrit (Hct Centrifuge and micro-haematocrit reader, Hawksley, UK) and haemoglobin via a standard cyanomethaemoglobin method using a spectrophotometer (Shimadzu 1240, Japan) applied to samples mixed with 5 ml Drabkin's reagent (GmbH Diagnostica, Boehringer Mannheim, Germany). From these data, changes in plasma volume were determined using the equations described by Dill & Costill (Dill and Costill 1974). The remaining EDTA-treated whole-blood

276 was then also centrifuged at 4000 *g* for 15 min at 4 ºC and before storage at -80 ºC pending later
277 analysis for vitamin C (after 1:1 dilution in 10% metaphosphoric acid; Sigma, UK) and vitamin
278 E using high-performance liquid chromatography (HPLC).
279
280 Plasma vitamin C concentrations were determined as in our previous studies (e.g.
281 Thompson et al. 2004), which involed separation using a 5 μm, 250 mm x 4.6 mm c18 Luna
282 column (Phenomenex, UK) with flow rate set at 1.2 $ml \cdot min^{-1}$ (producing a retention time of ~3.4
283 min) and using a degassed mobile phase of perchloric acid (Fischer Scientific, UK) adjusted to
284 pH 1.2 at room temperature; for analysis, plasma supernatants were diluted (1:1) in chilled 5%
285 metaphosphoric acid (Sigma, UK) and 50 μl used for injection via an autosaampler (Basic
286 Marathon, Spark, Netherlands). Spectrophotometric detection was then set at a wavelength of
287 241 nm (Pye, Unicam Ltd., UK) using a standard curve generated from ascorbic acid in the range
288 0-300 $\mu mol \cdot l^{-1}$. Similarly, plasma vitamin E (α-tocopherol) concentrations were determined
289 according to the methods described by Hess et al. (Hess et al. 1991). This involved separation
290 using a 5 μm, 250 mm x 4.6 mm Beckman Ultrasphere ODS column (Beckman, High Wycombe,
291 UK) set at 28ºC with flow rate set at 1.5 $ml \cdot min^{-1}$ (producing a retention time of ~7.2 min). The
292 mobile phase was acetonitrile:tetrahydrofurane:methanol:BHT-ammonium acetate
293 (684:220:68:28). Prior to analysis, vitamin E was extracted using hexane (containing 500 mg
294 $BHT \cdot {}^{L-}1$) and rapidly dried. The dry sample was dissolved in 200 μl of 1,4
295 dioxan:ethanol:acetonitrile (20:20:40 by volume) and shaken for 5-10 min before injection.
296 Detection was by fluorescence using excitation/emission of 298/328 nm (Waters 470 scanning
297 fluorescence detector, Water, Watford, UK) using a standard curve generated from α-tocopherol
298 in the range 0-100 $\mu mol \cdot l^{-1}$
299
300 Lastly, the concentration of 8-isoprostane $F_2\alpha$ (F_2-isoprostanes) in urine samples was
301 determined via commercially available monoclonal antibody-based competitive Dissociation
302 Enhanced Lanthanide Fluoro Immuno Assay (AutoDELFIA 1235 Automatic immunoassay

system Perkin Elmer Life & Analytical Sciences, UK). In brief, this involved all urine samples being vortexed and then allowed to stand to remove precipitates, before samples were added to an anti-mouse plate pre-washed with a dissociation enhanced lanthanide fluorescence immunoassay. Both an anti- F_2-isoprostane monoclonal antibody and a tracer (8-iso-PGF2-ovalbumin-europium chelate) were then diluted in assay buffer for analysis.

Statistical Analyses

A two-way mixed-model analysis of variance (TreatmentxTime) was used to explore differences in the response of each group, with repeated-measures effects adjusted using the Greenhouse-Geisser correction for epsilon <0.75 and the Huynh-Feldt correction adopted for less severe asphericity. Where significant *F* values were identified for either Time or TreatmentxTime, the Holm-Bonferroni step-wise correction was applied to locate variance at each time-point relative to baseline and between treatments using paired and independent t-tests, respectively (Atkinson 2002). In addition, simple summary statistics were calculated in relation to the change from baseline to peak response, thus informing interesting questions related to the total magnitude of effect with each treatment (Hopkins et al. 2009; Matthews et al. 1990). Sample size estimations were based upon our previous investigation in which vitamin C supplementation resulted in a significantly improved recovery of peak isometric torque in the knee flexors relative to a placebo (Effect Size ~1.3; Thompson et al. 2001). We therefore estimated that a total sample size of ~40 would be required to provide ~90% power of detecting such an effect at an alpha level of $P \leq 0.05$ using the above statistical analyses. All data are expressed as mean ± standard deviation.

RESULTS

Antioxidants

Plasma vitamin C concentrations were higher following combined vitamin C & E supplementation, both relative to pre-supplemented levels (Time: F = 131, $P < 0.001$) and in

relation to the change observed over time in the placebo group (Interaction: F = 17, $P < 0.001$). Notably, there were significant differences between treatment groups at every time-point from pre-exercise to the final sample of recovery (Figure 1; $P \leq 0.003$). Unlike vitamin C, plasma vitamin E is more resistant to acute changes with exercise and so was not measured throughout trials. However, the response of vitamin E pre-post supplementation did differ between treatments (Interaction: F = 10, $P = 0.003$) such that pre-exercise concentrations were significantly higher in the group supplemented with vitamin C & E than the placebo group (11.3 ± 3.2 *versus* 8.0 ± 4.6 $\mu mol \cdot l^{-1}$; $P = 0.02$). Serum uric acid concentrations gradually increased during exercise and then decreased during recovery (Time: F = 10, $P < 0.001$), with no differences in this response between treatment groups at any time-point (data not shown).

Oxidative/Metabolic Stress

There were similar concentrations of F_2-isoprostanes in urine samples of both groups during supplementation and over the first 24 h of post-exercise recovery (Figure 2). However, there were differences in the decline in concentrations thereafter (Interaction: F = 0.9, $P = 0.04$). The overall change score from baseline to post-exercise nadir values therefore also approached significance ($P = 0.08$), although the tendency for slightly higher concentrations in the placebo group at baseline precluded establishment of any significant differences at the final recovery time-point *per se*. Likewise, serum cortisol responses also exhibited a different time-course for each treatment (Figure 3; Interaction: F = 3.1, $P = 0.05$), again with no statistical differences between treatments at any time-point but a significant effect of treatment in relation to the change from pre-exercise to peak values ($P = 0.02$).

Indices of Inflammation

The serum concentration of interleukin-6 increased dramatically in response to exercise following both treatments (Time: F = 16, $P < 0.001$). Although there was no significant

356 interaction between treatment and time (Interaction: F = 1.6, P = 0.3), a trend was apparent for
357 higher interleukin-6 concentrations after 1 h of recovery in the group supplemented with vitamin
358 C & E than the placebo group (Table 1; P = 0.05). All other inflammatory markers are
359 summarised alongside interleukin-6 in Table 1; there were no differences between treatments.
360
361 **Indices of Muscle Damage**
362 Exercise markedly increased serum myoglobin concentration (Time: F = 35, P < 0.001)
363 and creatine kinase activity (Time: F = 23, P < 0.001), with peak values occurring after 1h and
364 24 h of recovery, respectively (Figure 4 a/b). These responses were very similar in both time-
365 course and magnitude between treatments, with values remaining significantly elevated relative
366 to baseline at every follow-up time-point ($P \leq 0.05$).
367
368 **Muscle Function**
369 Measures of peak isometric torque for both the knee flexors (Time: F = 37, P < 0.001)
370 and extensors (Time: F = 16, P < 0.001), along with the range of motion about this joint (Time: F
371 = 57, P < 0.001), were significantly reduced following exercise and remained below baseline
372 values under both treatments for at least 168 h (Table 2; $P \leq 0.01$). This pattern of responses did
373 not differ between treatment groups.
374
375 **Muscle Soreness**
376 Participants' subjective ratings of perceived muscle soreness were not different between
377 treatment groups at any of the follow-up measurement time-points. The overall pattern across
378 both treatment groups was for pre-exercise ratings of 2 ± 1 to reach peak soreness ratings of 5 ±
379 2 after 24 h of recovery (Time: F = 29, P < 0.001), which remained at this same level at both
380 subsequent follow-up measurements (i.e. 36 and 48 h post-exercise).
381

382 **Additional Information**

383 Estimated changes in plasma volume were very similar between treatment groups in
384 response to supplementation, exercise and during recovery (Interaction: F = 0.3, $P = 0.9$). There
385 was a progressive increase in plasma volume over time relative to pre-supplemented values
386 across both groups (Time: F = 6.7, $P < 0.001$), with the percentage change significantly above
387 baseline after 24 h (5.5 ± 2.5%; $P = 0.01$) and 48 h (7.9 ± 11.5%; $P = 0.001$) of recovery.
388 Averaged across all measurements made during the exercise protocol, mean heart rate was 166 ±
389 14 beats·min^{-1} and the mean rating of perceived exertion (6-20 scale) was 14 ± 2, with both
390 variables well matched between treatment groups.

391

DISCUSSION

This study was designed to examine whether supplementation with mixed antioxidant (primarily vitamin C & E) supplements can reduce oxidative stress and facilitate recovery following exercise-induced muscle damage. Our original hypothesis was based upon the rationale that individual antioxidants such as vitamin C and E have been found to reduce oxidative stress, inflammation and/or have improved recovery of muscle function (Alessio et al. 1997; Cannon et al. 1991; Jakeman and Maxwell 1993; Meydani et al. 1993; Thompson et al. 2001). Therefore, it was reasonable to suggest that a mixed antioxidant supplement would not only combine such benefits but also facilitate interaction between different antioxidant mechanisms to attain a synergistic benefit. For example, while oxidation of the chromanol region of α-tocopherol can certainly delay oxidative membrane damage, this process itself transforms vitamin E into a free radical (tocopherolxyl) (Dekkers et al. 1996). This is of relevance as, in addition directly quenching free radicals, a related role of vitamin C may be to regenerate vitamin E back to its antioxidant form (tocopherol; Goldfarb 1993). However, notwithstanding the above rationale and contrary to our hypothesis, the present results suggest that there are no beneficial effects to be gained from supplementation with vitamin C & E in combination. Specifically, although exercise resulted in a marked increase in protein/enzyme leakage from skeletal muscle, initiation of inflammatory responses and impairment of muscle function, prior supplementation with these particular antioxidant vitamins did not attenuate any of these effects relative to placebo supplements.

Recovery of muscle function was employed as the primary outcome measure in this study given both the clear practical interpretation of resultant data and the relatively valid reflection of muscle damage provided (Clarkson and Hubal 2002). This is the first time that the efficacy of combined vitamin C & E supplementation has been assessed using this outcome measure in relation to exercise representative of that most commonly performed by athletes or habitual

418 exercisers. Nonetheless, the absence of any benefit associated with vitamin C & E
419 supplementation reported in the present study is consistent with two other studies that have
420 measured muscle function in recovery from either muscle-specific resistance exercise (Bloomer
421 et al. 2007) or ultra-endurance exercise (Mastaloudis et al. 2006) to evaluate similar doses of
422 combined vitamin C & E supplements. While it therefore remains a distinct possibility that these
423 findings may be specific to the precise antioxidant mixtures provided in these studies, it is
424 arguably less likely that alternative forms of exercise would reveal a positive effect of these
425 particular antioxidants supplements because they have now been shown to be equally ineffective
426 across all three exercise modes involving high load (i.e. resistance), high repetition (i.e.
427 endurance) and high frequency/rate (i.e. intermittent sprint) muscle actions.
428
429 The increase in plasma vitamin C concentrations that occurred in response to combined
430 vitamin C & E supplementation was elevated even further during exercise but only to a similar
431 extent as in the placebo group. This is in agreement with previous reports (Gleeson et al. 1987;
432 Thompson et al. 2001) and suggests that endogenous ascorbic acid release during exercise may
433 be independent of prior supplementation. Moreover, while the first hour of recovery saw further
434 increased plasma vitamin C concentrations only in the group supplemented with this vitamin,
435 subsequent measurements after 24 and 48 h of recovery revealed a progressive return to below
436 pre-exercise values despite continued supplementation over this period. Interestingly, an earlier
437 study to document a similar effect during recovery also found a strong positive correlation
438 between the plasma responses of vitamin C and cortisol to exercise, from which it was reasoned
439 that endogenous release of vitamin C from the adrenal glands may be directly associated with the
440 release of cortisol (Gleeson et al. 1987). This reasoning may therefore explain why cortisol
441 responses were lower for the group supplemented with combined vitamin C & E in the present
442 study and others that have supplemented with vitamin C alone (Peters et al. 2001; i.e. increased
443 vitamin C availability in other tissues may reduce adrenal secretion of both vitamin C and
444 cortisol).

445

446 Apart from the effects on plasma vitamin C and E and serum cortisol, the only other
447 outcome variables showing any differences between treatment groups were concentrations of F_2-
448 isoprostanes in urine and interleukin-6 in serum. The general pattern for greater responses of F_2-
449 isoprostanes and interleukin-6 to exercise after combined vitamin C & E supplementation is
450 inconsistent with all extant studies in this area, which have always reported oxidative stress and
451 inflammatory markers to be either lower (Bloomer et al. 2006; Fischer et al. 2004; Goldfarb et al.
452 2005; Machefer et al. 2007; Mastaloudis et al. 2004; Schroder et al. 2000) or unaffected
453 (Bloomer et al. 2007; Bloomer et al. 2006; Dawson et al. 2002; Kanter et al. 1993; Mastaloudis
454 et al. 2004; Petersen et al. 2001; Rokitzki et al. 1994; Teixeira et al. 2009). It is therefore
455 difficult to account for the present findings. One possibility is that the apparent effect in relation
456 to F_2-isoprostanes may represent a type I statistical error, particularly given the large inter-
457 individual variability for this measure, which questions whether a worthwhile difference between
458 treatment groups truly existed. Nevertheless, what is clear is that there was certainly no
459 reduction in the concentration of F_2-isoprostanes as was hypothesised. In contrast, the effect of
460 vitamin C & E supplementation on interleukin-6 concentrations after 1 h of recovery was far
461 more consistent between individuals in each group (Effect Size = 1.4) and is very similar in
462 magnitude (~80% higher than placebo) to existing data, albeit in the opposite direction (Fischer
463 et al. 2004; Thompson et al. 2001). The precise reason for these divergent findings clearly
464 requires further examination but might be explained by the fact that the supplements used in the
465 present study provided 60-100% more vitamin C (i.e. 800 $mg \cdot d^{-1}$) than previously reported (i.e.
466 400-500 $mg \bullet d^{-1}$; Fischer et al. 2004; Thompson et al. 2001). This is important in view of the
467 fact that vitamin C is a redox agent and, as such, has been found to operate as a prooxidant in
468 some circumstances (Carr and Frei 1999), with suggestions that the pharmacologic quantities of
469 vitamin C found in supplements (as opposed to foods) may have 'unbalanced biochemistry' that
470 actually favours free radical production (Herbert 1994).

471

One final point of note arising from this study relates to the plasma heat shock protein (HSP)70 concentrations, which were unaffected by vitamin C & E supplementation but increased from 1.2 ± 1.0 $ng \cdot ml^{-1}$ pre-exercise to a peak of 6.6 ± 5.7 $ng \cdot ml^{-1}$ immediately post-exercise (P = 0.003) when considered across both treatments. Importantly, systemic HSP70 may play an important signalling role between various tissues (Calderwood et al. 2007) and, to our knowledge, this is the first time that this variable has been reported in response to intermittent exercise. By comparison, plasma HSP70 responses to a typical ironman triathlon race or even just 45-60 min of treadmill running at various intensities/gradients can be between 2-4 times higher than the values reported here (Peake et al. 2005; Suzuki et al. 2006). To consider HSP70 within the context of the exercise protocol and intervention applied in the present study, it is interesting that one of the training adaptations to 6 weeks of intermittent running four times per week is an increased muscle protein content of HSP70 (Morton et al. 2009), which can also be achieved through dietary supplementation with 500 $mg \cdot d^{-1}$ of vitamin C for 8 weeks (Khassaf et al. 2003).

CONCLUSIONS

Within the context of the particular supplementation regimen and exercise stressors examined in the present study, there was no benefit of 6-weeks mixed antioxidant vitamin C & E supplementation in relation to recovery from exercise-induced muscle damage, although further investigations may be warranted to explore the unanticipated responses of interleukin-6 and cortisol. While it also remains to be determined whether other distinct antioxidant mixtures may confer the hypothesised effects under related conditions, the lack of beneficial effects reported here across a comprehensive and practically relevant range of outcome measures and in a relatively large cohort may question the need for future studies designed to evaluate the efficacy of similar vitamin C & E supplements for improving recovery in terms of muscle function and soreness.

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ETHICAL STANDARDS

This study complies with current UK laws pertaining to the conduct of scientific research.

CONFLICT OF INTEREST

Dr Hurst is employed by Unilever.

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Table 1: *Serum concentrations of selected inflammatory/stress markers prior to and during recovery from exercise after 6-wk mixed antioxidant vitamin C & E or placebo supplementation.*

	Exercise		Recovery		
	Pre	Post	1 h	24 h	48 h
Interleukin-6 ($pg \cdot ml^{-1}$)		#	*#		
Antioxidant (n = 9)	1.1 ± 0.7	11.8 ± 7.4	11.3 ± 3.4	2.8 ± 3.9	0.9 ± 0.4
Placebo (n = 9)	1.6 ± 1.7	9.5 ± 10.2	6.2 ± 3.8	2.1 ± 1.8	1.8 ± 2.7
Interleukin-1ra ($pg \cdot ml^{-1}$)		#	#		
Antioxidant (n = 20)	205 ± 109	559 ± 561	1523 ± 1402	270 ± 202	224 ± 107
Placebo (n = 18)	204 ± 70	435 ± 359	1137 ± 1055	247 ± 86	388 ± 655
C-reactive protein ($ng \cdot ml^{-1}$)					
Antioxidant (n = 7)	788 ± 551	416 ± 220	1024 ±1146	6431 ± 3256	2617 ± 1670
Placebo (n = 11)	2739 ± 3725	1842 ± 2925	1223 ± 720	4941 ± 5023	4740 ± 5116
TNF-α ($ng \cdot ml^{-1}$)					
Antioxidant (n = 19)	1.7 ± 1.5	1.7 ± 1.5	1.6 ± 1.3	1.4 ± 1.3	1.2 ± 0.6
Placebo (n = 16)	1.5 ± 1.1	1.6 ± 0.7	1.6 ± 0.7	1.5 ± 0.7	1.5 ± 0.7
HSP70 ($ng \cdot ml^{-1}$)		#	#		
Antioxidant (n = 20)	1.4 ± 1.2	6.6 ± 5.7	5.1 ± 4.2	1.1 ± 0.9	0.9 ± 0.7
Placebo (n = 18)	1.0 ± 0.7	6.5 ± 5.8	4.9 ± 4.3	1.0 ± 0.8	0.6 ± 0.5

** denotes time-point different between treatments (P = 0.05) without treatmentxtime interaction.*
 # denotes time-points different from baseline across both treatments (P ≤ 0.02).

660

661

Table 2: *Peak isometric torque of the knee extensors and flexors prior to and during recovery from exercise after 6-wk mixed antioxidant vitamin C & E or placebo supplementation.*

	Exercise		Recovery			
	Pre	Post	24 h	48 h	96 h	168 h
Flexion (N·m)		#	#	#	# ‡	# ‡
Antioxidant (n = 20)	203 ± 43	172 ± 43	170 ± 49	169 ± 41	168 ± 43	178 ± 40
Placebo (n = 18)	204 ± 47	169 ± 41	163 ± 43	163 ± 46	147 ± 21	158 ± 35
Extension (N·m)		#	#	#	# ‡	# ‡
Antioxidant (n = 20)	317 ± 69	269 ± 59	282 ± 66	281 ± 65	328 ± 71	336 ± 66
Placebo (n = 18)	308 ± 54	259 ± 46	276 ± 57	292 ± 57	298 ± 57	278 ± 73
Range of Motion (º)		#	#	#	# ‡	# ‡
Antioxidant (n = 20)	117 ± 8	106 ± 8	106 ± 10	105 ± 11	102 ± 5	108 ± 4
Placebo (n = 18)	120 ± 7	106 ± 6	105 ± 7	106 ± 10	102 ± 6	103 ± 3

 # denotes time-points different from baseline ($P \leq 0.01$).

‡ denotes n = 6 and n = 5 for antioxidant and placebo groups, respectively.

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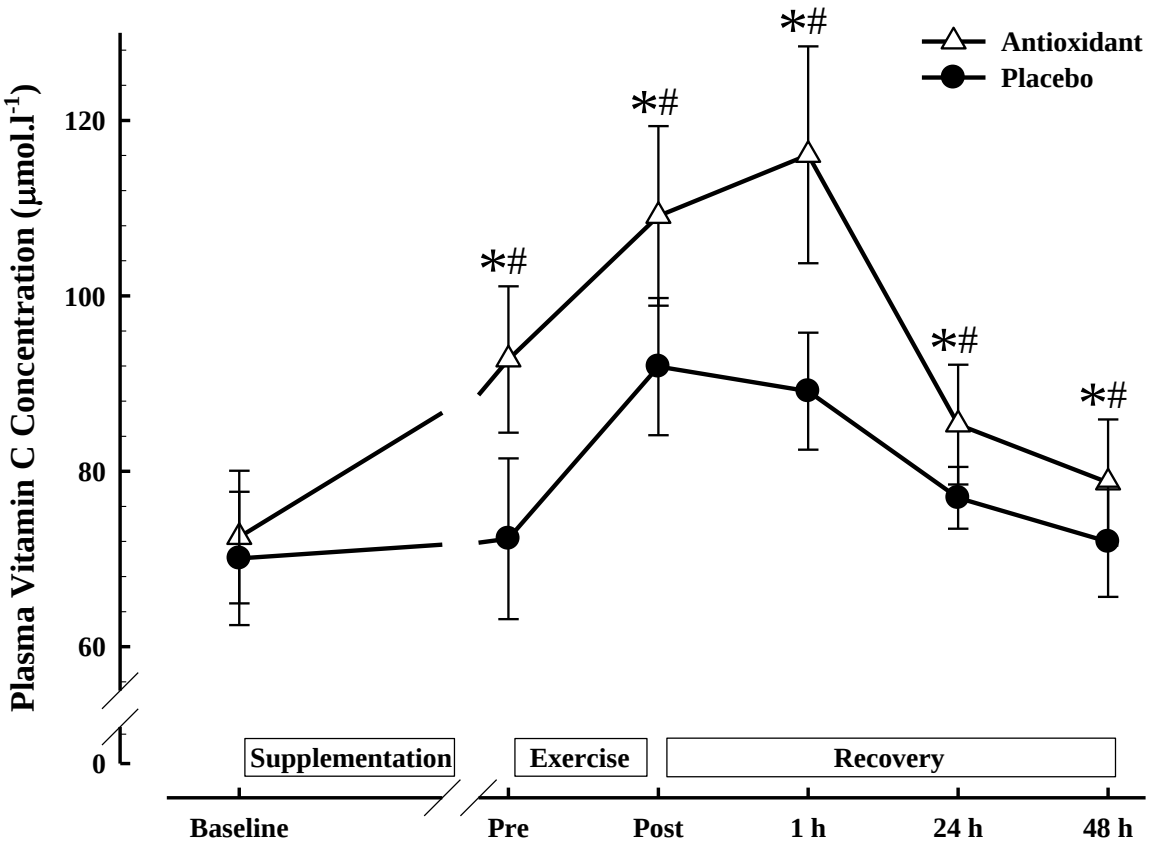
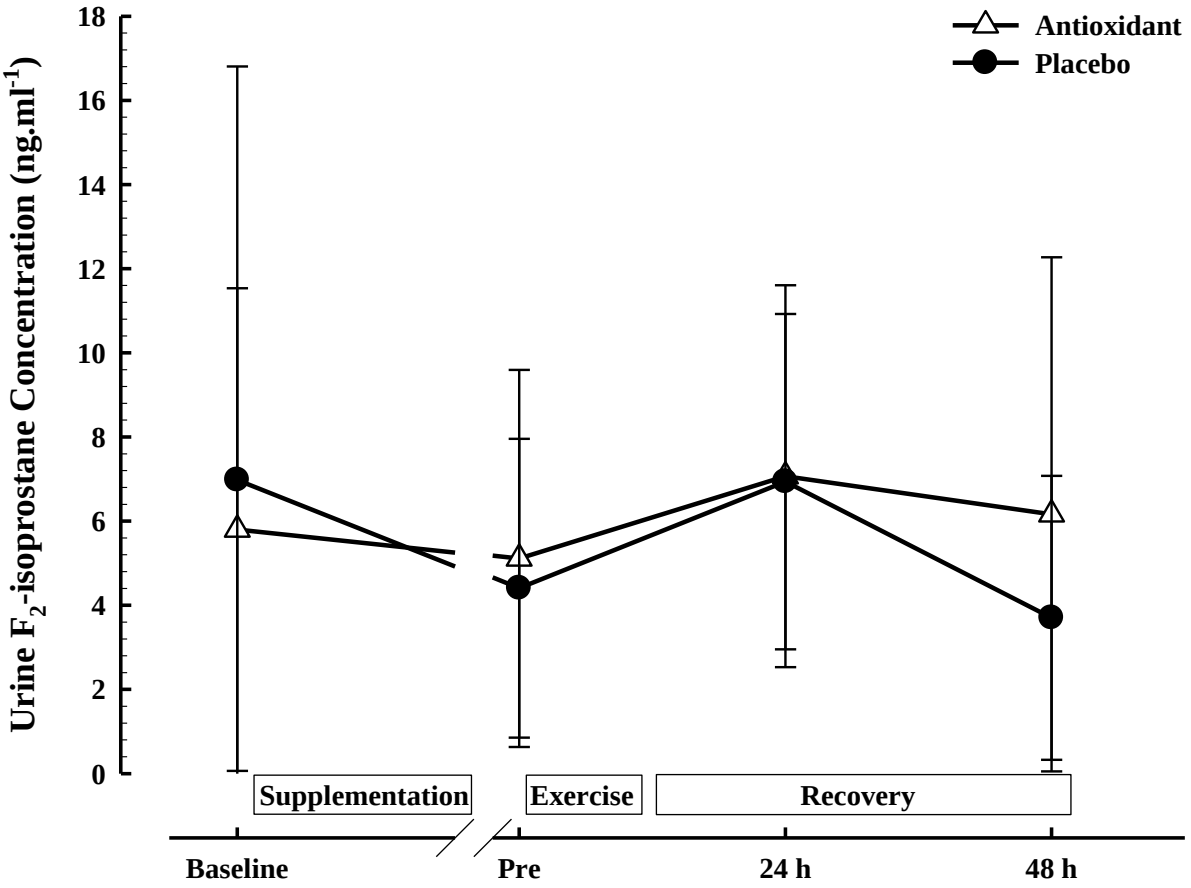


Fig. 1 Plasma vitamin C concentrations before and after 6-weeks mixed antioxidant vitamin C & E or placebo supplementation and then in recovery from exercise-induced muscle damage (Interaction: F = 17, $P < 0.001$). * denotes time-points different between treatments ($P \leq 0.003$); # denotes time-points different from baseline across both treatments ($P \leq 0.005$).



670

671 **Fig. 2** Urine F_2-isoprostane concentrations before and after 6-weeks mixed antioxidant vitamin

672 C & E or placebo supplementation and then in recovery from exercise-induced muscle damage

673 (Interaction: F = 0.9, *P* = 0.04).

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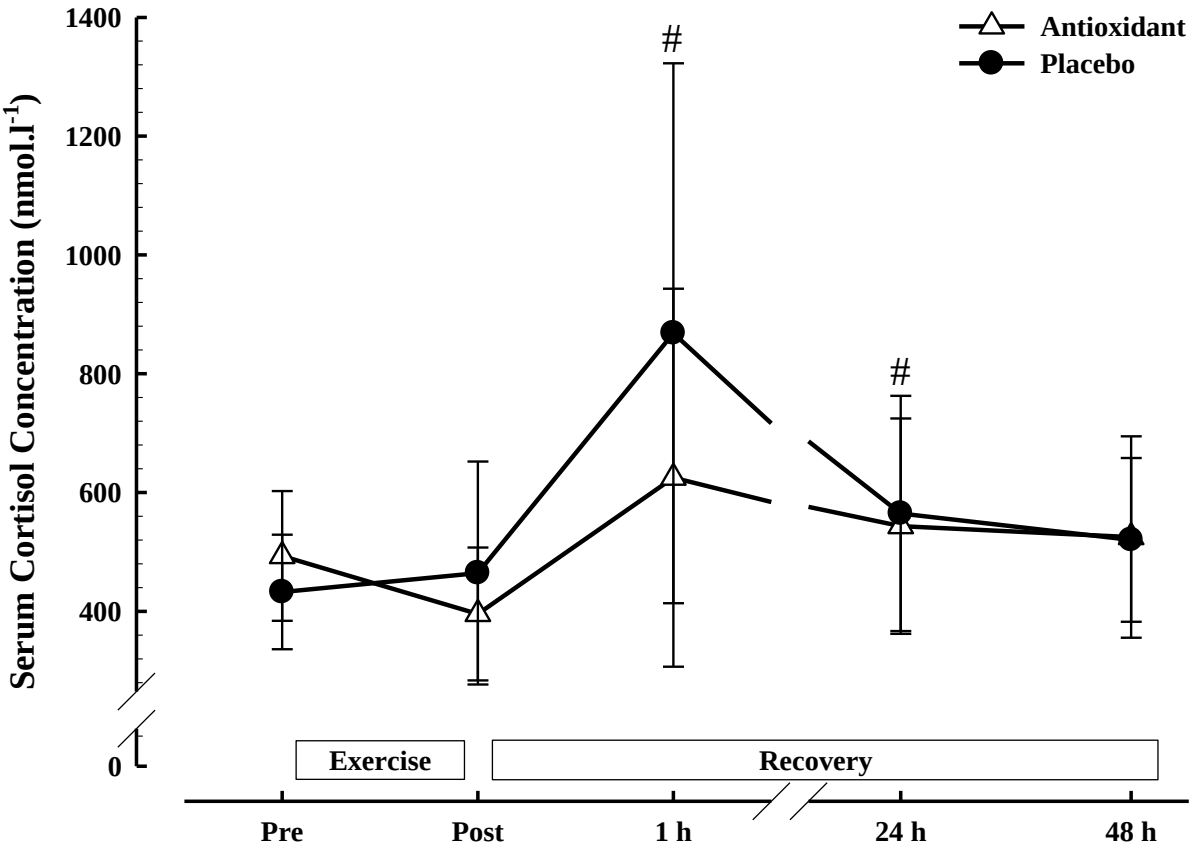

675

676 **Fig. 3** Serum cortisol concentrations prior to and during recovery from exercise following 6-
677 weeks mixed antioxidant vitamin C & E or placebo supplementation (Interaction: F = 3.1, *P* =
678 0.05). # denotes time-points different from baseline across both treatments ($P \leq 0.03$).

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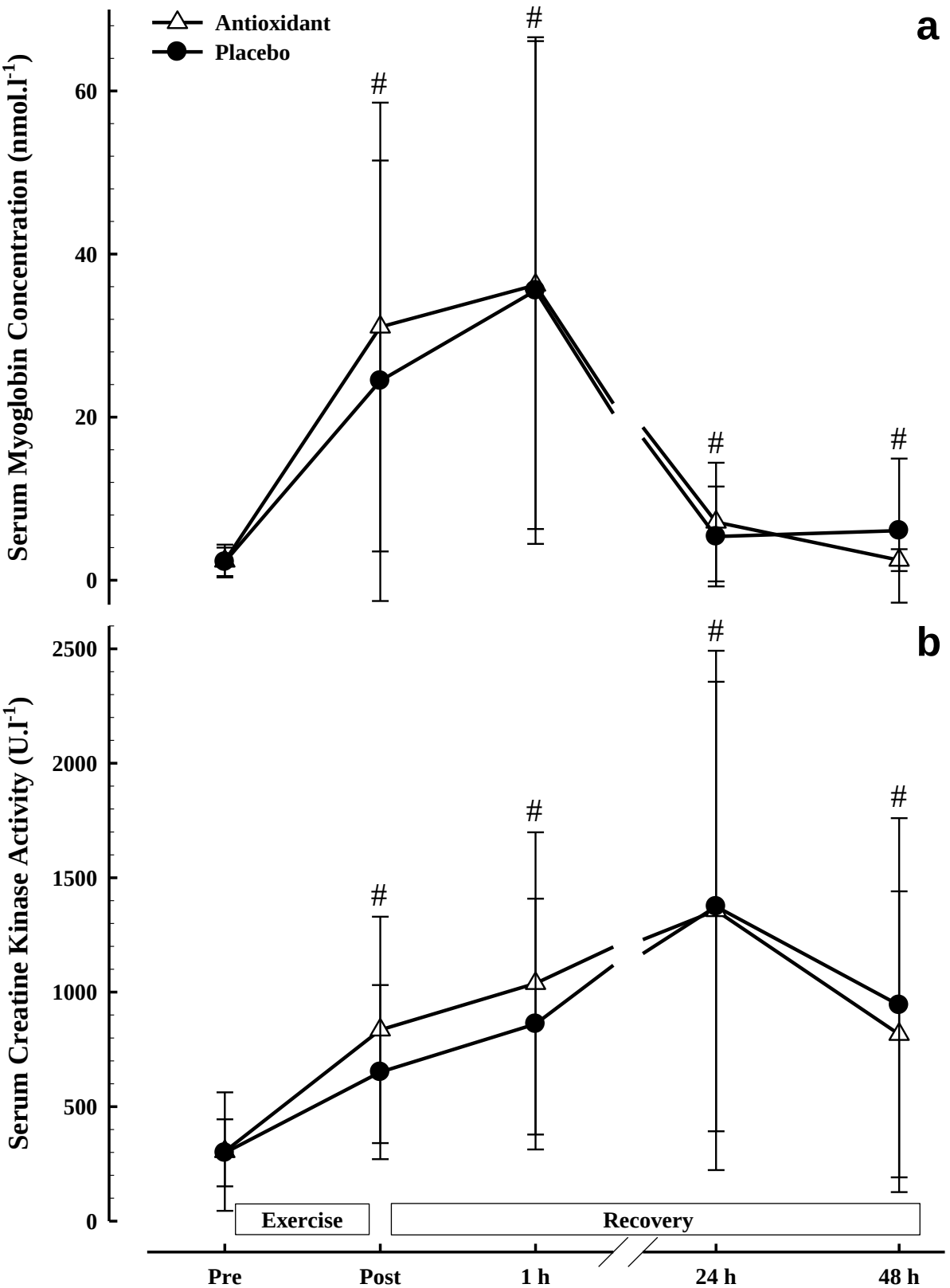

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681 **Fig. 4** Serum myoglobin concentrations (a) and creatine kinase activities (b) prior to and during
682 recovery from exercise following 6-weeks mixed antioxidant vitamin C & E or placebo
683 supplementation. # denotes time-points different from baseline across both treatments ($P \leq 0.05$).