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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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1 ABSTRACT

2 There is no consensus regarding the effects of mixed antioxidant vitamin C and/or vitamin E
3 supplementation on oxidative stress responses to exercise and restoration of muscle function.
4 Thirty-eight men were randomly assigned to receive either placebo group (n=18) or mixed
5 antioxidant (primarily vitamin C & E) supplements (n=20) in a double-blind manner. After 6-
6 weeks, participants performed 90 minutes of intermittent shuttle-running. Peak isometric torque
7 of the knee flexors/extensors and range of motion at this joint were determined before and after
8 exercise, with recovery of these variables tracked for up to 168 h post-exercise. Antioxidant
9 supplementation elevated pre-exercise plasma vitamin C ($93\pm 8 \mu\text{mol}\cdot\text{l}^{-1}$) and vitamin E (11 ± 3
10 $\mu\text{mol}\cdot\text{l}^{-1}$) concentrations relative to baseline ($P<0.001$) and the placebo group ($P\leq 0.02$). Exercise
11 reduced peak isometric torque (i.e. 9-19% relative to baseline; $P\leq 0.001$), which persisted for the
12 first 48 hours of recovery with no difference between treatment groups. In contrast, changes in
13 the urine concentration of F₂-isoprostanes responded differently to each treatment ($P=0.04$), with
14 a tendency for higher concentrations after 48 h of recovery in the supplemented group (6.2 ± 6.1
15 $\text{ng}\cdot\text{ml}^{-1}$ versus $3.7\pm 3.4 \text{ ng}\cdot\text{ml}^{-1}$). Vitamin C & E supplementation also affected serum cortisol
16 concentrations, with an attenuated increase from baseline to the peak values reached after 1 h of
17 recovery compared with the placebo group ($P=0.02$) and serum interleukin-6 concentrations
18 were higher after 1 h of recovery in the antioxidant group ($11.3\pm 3.4 \text{ pg}\cdot\text{ml}^{-1}$) than the placebo
19 group ($6.2\pm 3.8 \text{ pg}\cdot\text{ml}^{-1}$; $P=0.05$). Combined vitamin C & E supplementation did not reduce
20 markers of oxidative stress or inflammation nor did it facilitate recovery of muscle function after
21 exercise-induced muscle damage.

22

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

24 INTRODUCTION

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

43
44 Antioxidant vitamins are prime examples of such interventions. Our previous work
45 provided evidence for a modest beneficial effect of 2-weeks vitamin C supplementation in
46 relation to changes in muscle function/soreness and certain aspects of oxidative stress (i.e. lipid
47 peroxidation) and inflammation (i.e. cytokine response) after intermittent exercise (Thompson et
48 al. 2001). This benefit of vitamin C alone is consistent with other studies that have observed
49 improved recovery of maximum voluntary contraction (Jakeman and Maxwell 1993) and

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

60

61 In addition to the effects of ingesting single antioxidants, more recent studies have
62 explored the potential benefits of mixed antioxidants that are popular commercial products and
63 may offer better synergism between various interrelated antioxidant networks *in vivo* (Powers et
64 al. 2010). Most commonly, vitamin C (ascorbic acid) and vitamin E (α -tocopherol) have been
65 co-ingested (Bloomer et al. 2007; Bloomer et al. 2006; Dawson et al. 2002; Fischer et al. 2004;
66 Howatson et al. 2009; Mastaloudis et al. 2004; Mastaloudis et al. 2006; Petersen et al. 2001;
67 Rokitzki et al. 1994), although others have examined combined vitamin C and E supplements
68 that also include smaller quantities of vitamin A (α -/ β -carotene) precursors (Kanter et al. 1993;
69 Machefer et al. 2007; Schroder et al. 2000) or selenium (Goldfarb et al. 2005), among other
70 ingredients (i.e. lutein, zinc and magnesium; Teixeira et al. 2009). These supplements have been
71 evaluated using different types of exercise and, while some studies have reported reduced
72 evidence of oxidative stress associated with vitamin C & E supplementation (Bloomer et al.
73 2006; Fischer et al. 2004; Goldfarb et al. 2005; Machefer et al. 2007; Mastaloudis et al. 2004;
74 Schroder et al. 2000), others report no difference relative to placebo supplementation (Bloomer
75 et al. 2007; Bloomer et al. 2006; Dawson et al. 2002; Kanter et al. 1993; Rokitzki et al. 1994;
76 Teixeira et al. 2009). There appears to be more consistent results in relation to inflammatory

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

83

84 Notwithstanding the equivocal evidence regarding the effects of vitamin C & E
85 supplementation on oxidative stress and inflammation, direct histological examination of muscle
86 tissue via electron microscopy has not revealed any less ultrastructural disruption after exercise
87 when supplemented with these vitamins (Dawson et al. 2002). This finding is entirely consistent
88 with every study to have reported blood-borne biomarkers (e.g. creatine kinase, myoglobin and
89 lactate dehydrogenase) as proxy measures of muscle damage (Bloomer et al. 2007; Dawson et
90 al. 2002; Machefer et al. 2007; Mastaloudis et al. 2006; Petersen et al. 2001; Rokitzki et al. 1994;
91 Teixeira et al. 2009), none of which has reported significant protective effects of combined
92 vitamin C & E supplementation. From this perspective, it is noteworthy that such biomarkers do
93 not correlate well either with the magnitude of tissue injury (Komulainen et al. 1995) or
94 impairment of muscle function (Margaritis et al. 1999), the latter of which is believed to
95 represent the most valid indirect predictor of the degree of muscle damage sustained (Clarkson
96 and Hubal 2002). Combined with the fact that recovery of muscle function after exercise is also
97 of clear practical value, it is interesting that only two studies have monitored the response of this
98 variable to combined vitamin C & E intervention over the days following exercise (Bloomer et
99 al. 2007; Mastaloudis et al. 2006). Of these, neither reported any benefit of specific vitamin C &
100 E supplements in relation to either muscle-specific resistance exercise (Bloomer et al. 2007) or
101 ultra-endurance exercise (Mastaloudis et al. 2006). However, these exercise models may not
102 reflect the muscle damage typically experienced across a range of muscle groups during more
103 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

120 **MATERIALS AND METHODS**

121 **Approach to the Research Question**

122 The primary purpose of this study was to examine whether vitamin C & E
123 supplementation presents any benefit in terms of facilitating functional recovery following
124 exercise-induced muscle damage. Given the inherent inter-individual variance associated with
125 many measures of exercise-induced muscle damage, an ideal approach is to adopt a repeated-
126 measures cross-over design when assessing the efficacy of nutritional interventions (Betts et al.
127 2009) and one study has employed this approach in relation to combined vitamin C & E
128 supplements (Dawson et al. 2002). However, in view of the slow intracellular turnover of
129 vitamin E in many tissues (i.e. adipose tissue, adrenal glands, liver and skeletal muscle fibres;
130 Packer 1992), it was decided that an independent-measures 2-group design would be most
131 appropriate for this study but with a larger sample size (n = 18 & 20) than has previously been
132 used in this type of study (typically n = 10) in order to account for individual differences.

133
134 This relatively large cohort of participants were randomly assigned to receive either a
135 placebo supplement or a mixed antioxidant (primarily vitamin C & E) supplement for a period of
136 6 weeks prior to completing a high-intensity intermittent shuttle-running protocol. The use of
137 this protocol both increases ecological validity with specific reference to participation in team-
138 sports and also has the potential to induce substantial muscle damage on a whole-body level
139 through the combination of eccentrically-biased muscle actions (e.g. decelerations from repeated
140 sprints and frequent changes of direction) and high metabolic demand (Thompson et al. 1999).
141 Furthermore, as has recently been advocated (Powers et al. 2010), the antioxidant mixture
142 provided in this study was a cocktail including not only vitamins C and E but also lesser
143 quantities of other nutrients (detailed under ‘Supplementation’) that act synergistically with more
144 potent or direct antioxidants to protect against oxidative damage. Obviously, should any

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

147
148 We employed multiple outcome measures to provide a more valid overall interpretation
149 than would be possible using any one in isolation. In particular, serial assessments of muscle
150 function for up to a week following exercise represents a central novel element of this study,
151 while also affording high levels of both internal and external validity (Clarkson and Hubal 2002).
152 Furthermore, the measures of oxidative stress most commonly used in this area (e.g.
153 thiobarbituric acid adducts/malondialdehyde) are not specific to oxidative processes (Halliwell
154 and Chirico 1993), whereas urinary F₂-isoprostanes are more reliable biomarkers of lipid
155 peroxidation *in vivo* (Liu et al. 2009; Powers et al. 2010) and so were used in the present study.
156 For completeness and to facilitate comparisons with existing research on this topic, indirect
157 markers of skeletal muscle damage and inflammation were also determined in venous blood
158 samples collected over the hours and days following exercise-induced muscle damage.

159

160 **Participants**

161 Thirty-eight healthy young men volunteered to take part in this study and their
162 characteristics were as follows: age 22 ± 1 yr, body mass (BM) 77.0 ± 8.9 kg (beam balance
163 scales, Avery Ltd), height 1.78 ± 0.06 m (Stadiometer, Holtain Ltd), Body Mass Index 24 ± 2
164 $\text{kg}\cdot\text{m}^{-2}$, Σ triceps, biceps, subscapular and suprailiac skin-folds 34 ± 9 mm (callipers, Salter;
165 ACSM 1995) and $\dot{V}\text{O}_{2\text{max}}$ 55 ± 5 $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ (means \pm SD). All participants were non-
166 smokers and habitually active in a variety of sports but were unfamiliar with the specific exercise
167 protocol involved in this investigation. Once fully briefed regarding the nature of the study both
168 verbally and in writing, each participant provided written informed consent in keeping with the
169 requirements of the Loughborough University Ethical Advisory Committee who approved this
170 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: $13381 \pm 3940 \text{ kJ}\cdot\text{d}^{-1}$, $54 \pm 7\%$
178 carbohydrate, $30 \pm 6\%$ fat, $16 \pm 4\%$ protein, vitamin C $123 \pm 79 \text{ mg}\cdot\text{d}^{-1}$, vitamin E 7.7 ± 2.9
179 $\text{mg}\cdot\text{d}^{-1}$ *versus* Antioxidant: $13415 \pm 2935 \text{ kJ}\cdot\text{d}^{-1}$, $53 \pm 5\%$ carbohydrate, $31 \pm 6\%$ fat, $16 \pm 3\%$
180 protein, vitamin C $120 \pm 30 \text{ mg}\cdot\text{d}^{-1}$, vitamin E $8.7 \pm 4.6 \text{ mg}\cdot\text{d}^{-1}$; mean \pm 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 NORMTM isokinetic dynamometer (model 770), thus
192 establishing sufficiently reliable baseline measurements of peak isometric torque in knee flexors
193 (co-efficient of variation = $6 \pm 4\%$) and extensors (co-efficient of variation = $8 \pm 5\%$) 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 sagittal 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 $1.05 \text{ rad}\cdot\text{s}^{-1}$. 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 105° relative to full extension) at knee angles of 60° and 20° 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 ($5 \text{ mL}\cdot\text{kg BM}^{-1}$) 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 $\dot{V}\text{O}_{2\text{max}}$ (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 ($2 \text{ mL}\cdot\text{kg BM}^{-1}$) of plain water were ingested. Heart-rate (Sports Tester PE3000,

223 Polar, Finland) and ratings of perceived exertion (Borg 1973) were measured at regular intervals
224 during exercise. Ambient temperature and humidity were closely matched between treatments
225 (Placebo: $16 \pm 1^\circ\text{C}$, $54 \pm 6\%$ *versus* Antioxidant: $16 \pm 1^\circ\text{C}$, $55 \pm 6\%$).

226
227 Immediately after exercise, a venous blood sample was drawn by venepuncture followed
228 by an assessment of muscle function. Thereafter, participants rested in the laboratory for 1 h
229 before a further follow-up blood sample was obtained by venepuncture. Supplements ingested
230 on test days were taken only after this final blood sample had been consumed, coincident with
231 the participants' next meal. Approximately (± 2 h) 24 and 48 h following the cessation of
232 exercise, participants returned to the laboratory to provide venous blood and urine samples,
233 along with measures of muscle function. Ratings of perceived muscle soreness were again
234 obtained from participants at these time points and at 12 h intervals in between visits. A sub-
235 population of the total cohort ($n = 11$) also provided additional follow-up measurements of
236 muscle function after 96 and 168 h of recovery.

237

238 **Supplementation**

239 The antioxidant and placebo supplements were randomly assigned in a double-blind
240 manner in the form of identical capsules, ingested twice daily with water following meals. Each
241 antioxidant capsule provided 400 mg vitamin C (ascorbic acid), 268 mg vitamin E (RRR- α -
242 tocopherol), 2 mg vitamin B6 (pyridoxine hydrochloride), 200 μg vitamin B9 (folic acid), 5 μg
243 zinc sulphate monohydrate and 1 μg Vitamin B12 (cyanocobalamin), while placebo capsules
244 contained only lactose (RP Scherer Limited, UK). When considered within the context of
245 participants' habitual diets, antioxidant supplementation therefore resulted in approximately an
246 8-fold increase in daily vitamin C intake and approximately a 160-fold increase in daily vitamin
247 E intake. This therefore represents $>1000\%$ and $\sim 3600\%$ of the recommended daily allowance
248 for vitamin C and E, respectively, but well within the upper limit that poses risk of serious

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

254

255 **Sampling and Analyses**

256 From each 8 mL blood sample, 4 mL was dispensed into a non-anticoagulant tube where
257 it was left to clot for 45 min at room temperature and then centrifuged at 4000 g for 15 min at 4
258 °C. The serum fraction was then stored at -80 °C pending analyses at 37°C using commercially
259 available enzymatic colorimetric assays for myoglobin, creatine kinase and uric acid (Randox,
260 UK) and an automated spectrophotometric analyser (COBAS-Mira plus, Roche) and for cortisol
261 via radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, UK) and an automated
262 gamma counter (Cobra II, Packard Instruments Company Inc, US). Where sufficient serum was
263 available for each participant, concentrations of interleukin-6 (R&D Systems Inc. UK),
264 interleukin-1 receptor antagonist (R&D Systems Inc. UK), C-reactive protein (DSL, UK), heat
265 shock protein (HSP)70 (Stressgen Biotechnologies Inc. USA) and tumor necrosis factor (TNF)- α
266 (R&D Systems Inc. UK) were determined using commercially available Enzyme-Linked
267 ImmunoSorbent Assays (ELISA) with a spectrophotometric plate reader (Dynex Technologies
268 Inc. USA). The remaining 4 mL of whole-blood was transferred into a tube containing the
269 anticoagulant ethylenediaminetetraacetic acid (EDTA), from which triplicate 50 μ l and 20 μ l
270 samples were taken for the respective manual determination of haematocrit (Hct Centrifuge and
271 micro-haematocrit reader, Hawksley, UK) and haemoglobin via a standard
272 cyanomethaemoglobin method using a spectrophotometer (Shimadzu 1240, Japan) applied to
273 samples mixed with 5 ml Drabkin's reagent (GmbH Diagnostica, Boehringer Mannheim,
274 Germany). From these data, changes in plasma volume were determined using the equations
275 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 °C and before storage at -80 °C 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 involved 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·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 autosampler (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 µmol·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 28°C with flow rate set at 1.5 ml·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·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 µmol·l⁻¹

299
300 Lastly, the concentration of 8-isoprostane F₂ α (F₂-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

303 system Perkin Elmer Life & Analytical Sciences, UK). In brief, this involved all urine samples
304 being vortexed and then allowed to stand to remove precipitates, before samples were added to
305 an anti-mouse plate pre-washed with a dissociation enhanced lanthanide fluorescence
306 immunoassay. Both an anti- F₂-isoprostane monoclonal antibody and a tracer (8-iso-PGF₂-
307 ovalbumin-europium chelate) were then diluted in assay buffer for analysis.

308

309 **Statistical Analyses**

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

325 **RESULTS**

326 **Antioxidants**

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

329 relation to the change observed over time in the placebo group (Interaction: $F = 17$, $P < 0.001$).
330 Notably, there were significant differences between treatment groups at every time-point from
331 pre-exercise to the final sample of recovery (Figure 1; $P \leq 0.003$). Unlike vitamin C, plasma
332 vitamin E is more resistant to acute changes with exercise and so was not measured throughout
333 trials. However, the response of vitamin E pre-post supplementation did differ between
334 treatments (Interaction: $F = 10$, $P = 0.003$) such that pre-exercise concentrations were
335 significantly higher in the group supplemented with vitamin C & E than the placebo group (11.3
336 ± 3.2 versus 8.0 ± 4.6 $\mu\text{mol}\cdot\text{l}^{-1}$; $P = 0.02$). Serum uric acid concentrations gradually increased
337 during exercise and then decreased during recovery (Time: $F = 10$, $P < 0.001$), with no
338 differences in this response between treatment groups at any time-point (data not shown).

339

340

341 **Oxidative/Metabolic Stress**

342 There were similar concentrations of F₂-isoprostanes in urine samples of both groups
343 during supplementation and over the first 24 h of post-exercise recovery (Figure 2). However,
344 there were differences in the decline in concentrations thereafter (Interaction: $F = 0.9$, $P = 0.04$).
345 The overall change score from baseline to post-exercise nadir values therefore also approached
346 significance ($P = 0.08$), although the tendency for slightly higher concentrations in the placebo
347 group at baseline precluded establishment of any significant differences at the final recovery
348 time-point *per se*. Likewise, serum cortisol responses also exhibited a different time-course for
349 each treatment (Figure 3; Interaction: $F = 3.1$, $P = 0.05$), again with no statistical differences
350 between treatments at any time-point but a significant effect of treatment in relation to the
351 change from pre-exercise to peak values ($P = 0.02$).

352

353 **Indices of Inflammation**

354 The serum concentration of interleukin-6 increased dramatically in response to exercise
355 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 $5 \pm$
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 ($5.5 \pm 2.5\%$; $P = 0.01$) and 48 h ($7.9 \pm 11.5\%$; $P = 0.001$) of recovery.
388 Averaged across all measurements made during the exercise protocol, mean heart rate was $166 \pm$
389 $14 \text{ beats}\cdot\text{min}^{-1}$ and the mean rating of perceived exertion (6-20 scale) was 14 ± 2 , with both
390 variables well matched between treatment groups.

391

392 **DISCUSSION**

393 This study was designed to examine whether supplementation with mixed antioxidant
394 (primarily vitamin C & E) supplements can reduce oxidative stress and facilitate recovery
395 following exercise-induced muscle damage. Our original hypothesis was based upon the
396 rationale that individual antioxidants such as vitamin C and E have been found to reduce
397 oxidative stress, inflammation and/or have improved recovery of muscle function (Alessio et al.
398 1997; Cannon et al. 1991; Jakeman and Maxwell 1993; Meydani et al. 1993; Thompson et al.
399 2001). Therefore, it was reasonable to suggest that a mixed antioxidant supplement would not
400 only combine such benefits but also facilitate interaction between different antioxidant
401 mechanisms to attain a synergistic benefit. For example, while oxidation of the chromanol
402 region of α -tocopherol can certainly delay oxidative membrane damage, this process itself
403 transforms vitamin E into a free radical (tocopherolxyl) (Dekkers et al. 1996). This is of
404 relevance as, in addition directly quenching free radicals, a related role of vitamin C may be to
405 regenerate vitamin E back to its antioxidant form (tocopherol; Goldfarb 1993). However,
406 notwithstanding the above rationale and contrary to our hypothesis, the present results suggest
407 that there are no beneficial effects to be gained from supplementation with vitamin C & E in
408 combination. Specifically, although exercise resulted in a marked increase in protein/enzyme
409 leakage from skeletal muscle, initiation of inflammatory responses and impairment of muscle
410 function, prior supplementation with these particular antioxidant vitamins did not attenuate any
411 of these effects relative to placebo supplements.

412

413 Recovery of muscle function was employed as the primary outcome measure in this study
414 given both the clear practical interpretation of resultant data and the relatively valid reflection of
415 muscle damage provided (Clarkson and Hubal 2002). This is the first time that the efficacy of
416 combined vitamin C & E supplementation has been assessed using this outcome measure in
417 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 F₂-
448 isoprostanes in urine and interleukin-6 in serum. The general pattern for greater responses of F₂-
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 F₂-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 F₂-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·d⁻¹) than previously reported (i.e.
466 400-500 mg·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

472 One final point of note arising from this study relates to the plasma heat shock protein
473 (HSP)70 concentrations, which were unaffected by vitamin C & E supplementation but increased
474 from $1.2 \pm 1.0 \text{ ng}\cdot\text{ml}^{-1}$ pre-exercise to a peak of $6.6 \pm 5.7 \text{ ng}\cdot\text{ml}^{-1}$ immediately post-exercise ($P =$
475 0.003) when considered across both treatments. Importantly, systemic HSP70 may play an
476 important signalling role between various tissues (Calderwood et al. 2007) and, to our
477 knowledge, this is the first time that this variable has been reported in response to intermittent
478 exercise. By comparison, plasma HSP70 responses to a typical ironman triathlon race or even
479 just 45-60 min of treadmill running at various intensities/gradients can be between 2-4 times
480 higher than the values reported here (Peake et al. 2005; Suzuki et al. 2006). To consider HSP70
481 within the context of the exercise protocol and intervention applied in the present study, it is
482 interesting that one of the training adaptations to 6 weeks of intermittent running four times per
483 week is an increased muscle protein content of HSP70 (Morton et al. 2009), which can also be
484 achieved through dietary supplementation with $500 \text{ mg}\cdot\text{d}^{-1}$ of vitamin C for 8 weeks (Khassaf et
485 al. 2003).

486

487 **CONCLUSIONS**

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

498

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501 Hext and Simon Jones for their assistance with data collection and Duncan Talbot for assistance
502 with sample analysis.

503

504 **ETHICAL STANDARDS**

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

506

507 **CONFLICT OF INTEREST**

508 Dr Hurst is employed by Unilever.

509

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657

658

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·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·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·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·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·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 treatment×time interaction.

denotes time-points different from baseline across both treatments ($P \leq 0.02$).

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.

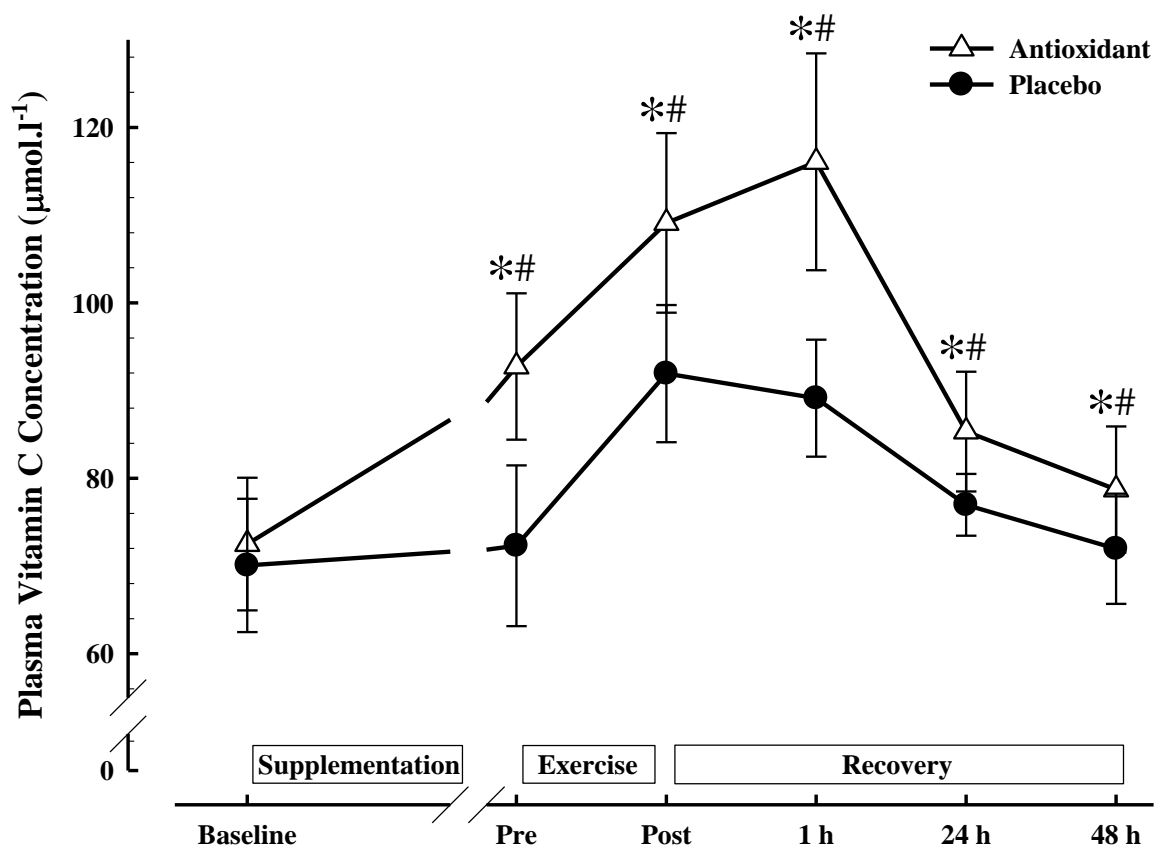
	Exercise			Recovery		
	Pre	Post	24 h	48 h	96 h	168 h
Flexion (N·m)		#	#	#	# ‡	# ‡
Antioxidant (n = 20)	203 ± 43	172 ± 43	170 ± 49	169 ± 41	168 ± 43	178 ± 40
Placebo (n = 18)	204 ± 47	169 ± 41	163 ± 43	163 ± 46	147 ± 21	158 ± 35
Extension (N·m)		#	#	#	# ‡	# ‡
Antioxidant (n = 20)	317 ± 69	269 ± 59	282 ± 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 (°)		#	#	#	# ‡	# ‡
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.

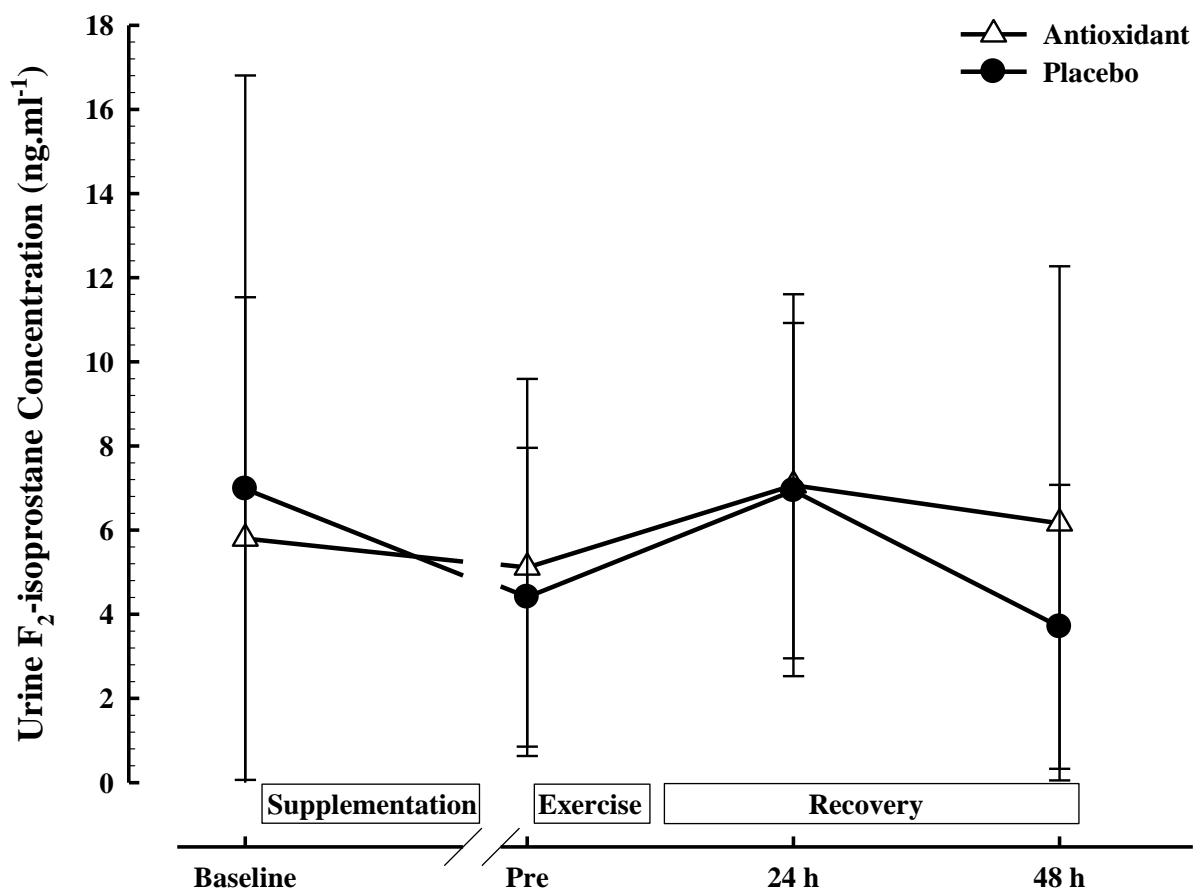
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664
 665 **Fig. 1** Plasma vitamin C concentrations before and after 6-weeks mixed antioxidant vitamin C &
 666 E or placebo supplementation and then in recovery from exercise-induced muscle damage
 667 (Interaction: $F = 17$, $P < 0.001$). * denotes time-points different between treatments ($P \leq 0.003$);
 668 # denotes time-points different from baseline across both treatments ($P \leq 0.005$).

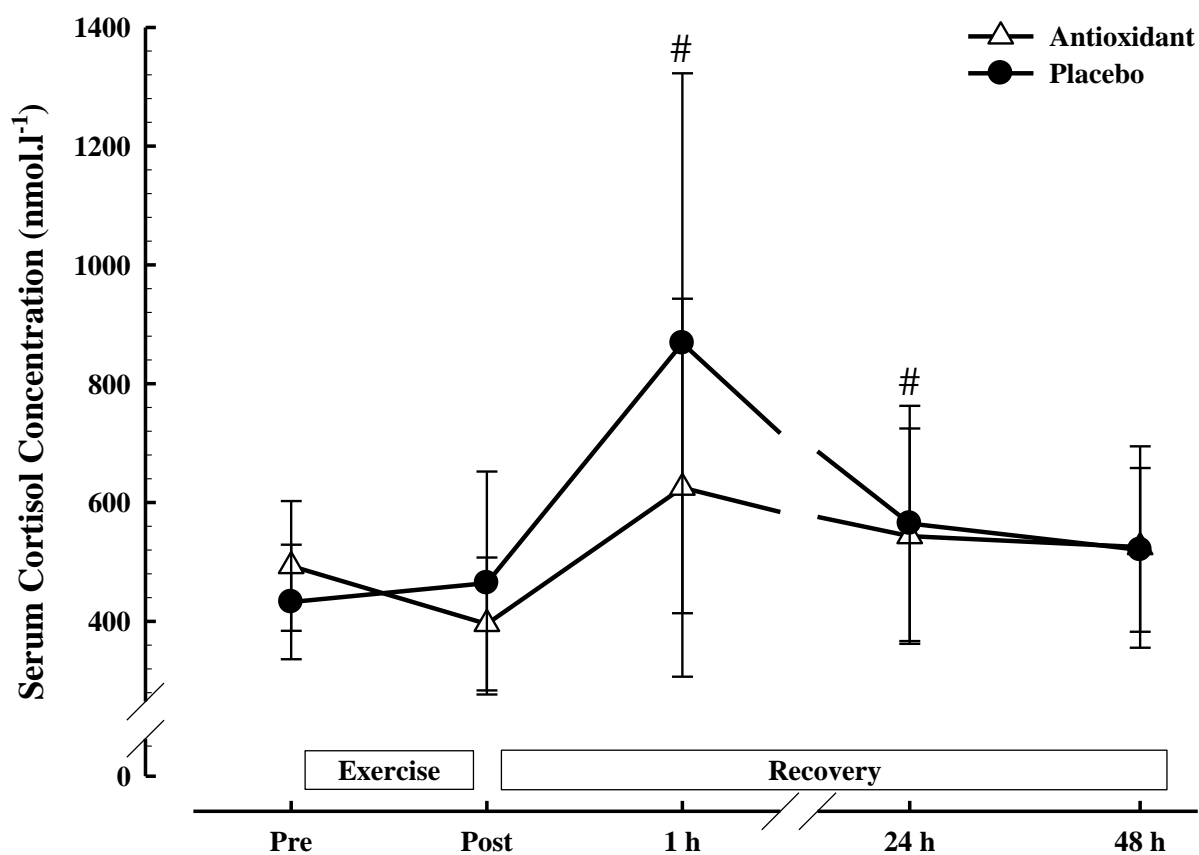
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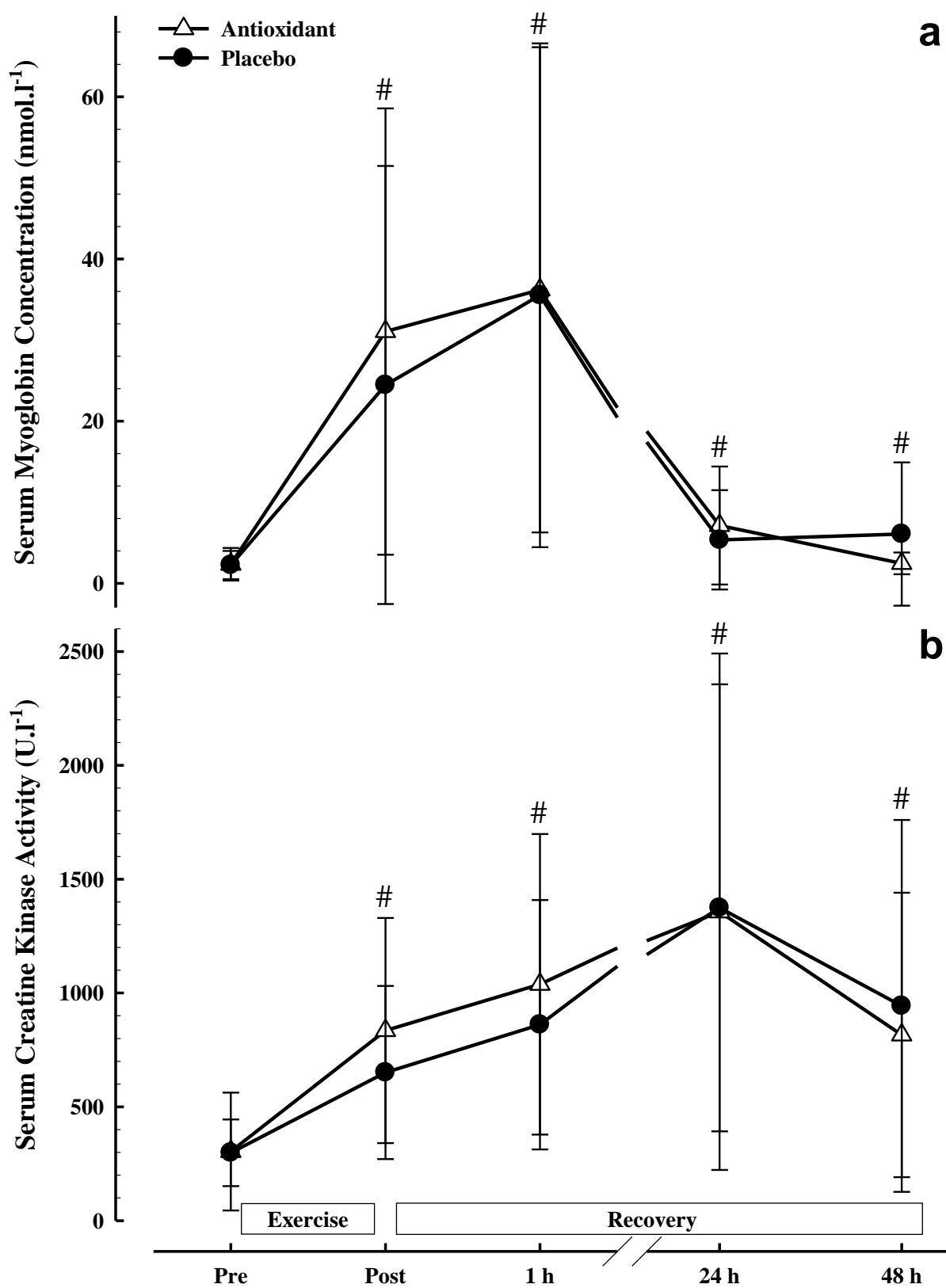
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671 **Fig. 2** Urine F₂-isoprostane concentrations before and after 6-weeks mixed antioxidant vitamin
 672 C & E or placebo supplementation and then in recovery from exercise-induced muscle damage
 673 (Interaction: $F = 0.9$, $P = 0.04$).

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 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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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 ($P \leq 0.05$).