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Antioxidant supplementation and exercise-induced oxidative stress in the 60-year-old as measured by antipyrine hydroxylates

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The effects of 12 weeks of antioxidant supplementation on exercise-induced oxidative stress were investigated in older adults (60 (SE 1) years; BMI 26 (SE 1) kg/m^2). Subjects were randomly divided in two groups: supplementation (*n* 11) with 100 mg DL-α-tocopheryl acetate, 200 mg ascorbic acid, and 2 mg β-carotene, and placebo (*n* 9). Before and after the 12 week supplementation period, subjects cycled for 45 min at submaximal intensity (50 % maximal workload capacity). Antipyrine was used as marker for oxidative stress. Antipyrine reacts quickly with hydroxyl radicals to form *para*- and *ortho*-hydroxyantipyrine. The latter metabolite is not formed in man through the mono-oxygenase pathway of cytochrome P450. Daily supplementation significantly increased plasma concentrations of α-tocopherol and β-carotene in the supplemented group (Δ 14·4 (SE 3·2) and 0·4 (SE 0·1) μmol/l; $P<0{\cdot}001$ and $P<0{\cdot}01$). No significant differences, within and between groups, were observed in the exercise-induced increase in the ratios *para*- and *ortho*-hydroxyantipyrine to antipyrine. In addition, supplementation did not affect the exercise-induced increase in thiobarbituric acid reactive substances in plasma. In conclusion, in 60-year-old subjects antioxidant supplementation had no effect on the exercise-induced increase in oxidative stress as measured by free radical products of antipyrine.

Ageing: Antipyrine: Free radicals: Antioxidant vitamins

It has been hypothesized that ageing is associated with deleterious effects of reactive oxygen species taking place throughout the lifespan. There is strong evidence that reactive oxygen species play an important role in many degenerative diseases like cancer, atherosclerosis and diabetes (Beckman & Ames, 1998). The effects of reactive oxygen species are scavenged by antioxidant enzymes, as well as with low-molecular-mass non-enzymatic antioxidant vitamins. In cell membranes the most important is α-tocopherol, the major member of the vitamin E family. This molecule acts as a chain-breaking antioxidant, intercepting lipid peroxyl radicals and so terminating lipid peroxidation (Brigelius-Flohé & Traber, 1999). Other lipid-soluble compounds that can act as antioxidants are the carotenoids, such as β-carotene (Bast *et al.* 1998). The major water-soluble free radical scavenger is ascorbic acid (vitamin C), which also plays a role in sparing vitamin E by regenerating α-tocopherol from the oxidized tocopheroxyl radical (Packer *et al.* 1979; Sies & Stahl, 1995). Several studies in elderly human subjects have shown that antioxidant supplementation resulted in an improved immune function and a decreased oxidative damage (Meydani *et al.* 1993; Hughes, 1999; Pallast *et al.* 1999).

Besides antioxidant supplementation, exercise is also often prescribed to older individuals to achieve optimal health. It is, however, generally known that exercise increases reactive oxygen species generation and results in an induction of the antioxidant defence system (Davies *et al.* 1982; Powers *et al.* 1994; Ji, 1999). Recently, Coolen (2000) observed no increase in oxidative stress in young adults, who cycled for 2 h at 50 % maximal workload capacity

Abbreviations: *o*-APOH, *ortho*-hydroxyantipyrine; *p*-APOH, *para*-hydroxyantipyrine; TBARS, thiobarbituric acid reactive substances; W_{max}, maximal loading capacity.

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(W_{max}). It was proposed that the training status of the subjects was too high to observe an effect on exercise-induced oxidative stress at such a moderate intensity. In older subjects, however, we observed that submaximal exercise resulted in a significant increased oxidative stress (Meijer *et al.* 2001). Furthermore, we showed that training did not affect the exercise-induced increase in oxidative stress. Because ageing is associated with an increased susceptibility to free radical damage, it would be informative to know whether physically-active older adults would benefit from an antioxidant vitamin supplementation intervention.

To measure oxidative stress *in vivo*, antipyrine (2,3-dimethyl-1-phenyl-3-pyrazoline-5-one), an exogenous marker, was used. The properties of antipyrine make it a suitable marker for measuring oxidative stress *in vivo*. Following oral ingestion, antipyrine is completely absorbed and uniformly distributed in the total body water after approximately 1 h (Siri, 1956). In addition, antipyrine is independent of blood flow to the liver, which is an advantage in clinical studies where blood flow is altered, e.g. exercise experiments (Hartleb, 1991). Moreover, the reaction rate constant of antipyrine with hydroxyl radicals, the most aggressive free radicals that exist, is in the order of 10^{10} l/mol per s (Forni *et al.* 1988). Exposure of an antipyrine solution in water to ^{60}Co γ-radiation leads to the formation of three phenolic antipyrine derivatives: *para*-hydroxyantipyrine (*p*-APOH), *ortho*-hydroxyantipyrine (*o*-APOH) and *meta*-hydroxyantipyrine. The latter two metabolites are not endogenously formed (Coolen *et al.* 1997). Recently, we have shown that a submaximal bout of cycling exercise in elderly subjects resulted in a significant increase in the plasma levels of *p*- and *o*-APOH (Meijer *et al.* 2001).

So far, studies in the elderly that have examined the effect of an antioxidant vitamin supplementation intervention on exercise-induced oxidative stress have only relied on endogenous markers. Therefore, the purpose of the present study was to investigate the effects of 12 weeks of antioxidant supplementation on exercise-induced oxidative stress in 60-year-old human subjects by using antipyrine as an exogenous marker.

Materials and methods

Study design

The study was designed as a placebo-controlled, double-blind, randomized, parallel group trial. Before and after the 12 week supplementation period, subjects performed a maximal exercise test and a 45 min cycling test at submaximal intensity. Exercise-induced oxidative stress was measured during the second cycling test by using antipyrine.

Subjects

Twenty-two healthy men and women aged ⩾55 years, with no known medical illness and receiving no prescription medication, participated in the study. Subjects were recruited from advertisements in the local media, and were randomly divided over two groups: eleven subjects in the supplementation group and eleven subjects in the control placebo group. Two subjects dropped out of the study due to personal reasons. Final data processing was done with nine subjects in the placebo group. Subject characteristics are shown in Table 1. Detailed information concerning the purpose and methods used in the study was provided before informed consent was obtained. The local Ethical Committee approved the study.

Protocol

After an overnight stay at the laboratory, W_{max} and maximal O_2 uptake were measured, on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) during an incremental exercise test, as described before (Meijer *et al.* 1999). After 1 h a Teflon catheter (Quick Cath®II; Baxter Healthcare S.A., Swinford, Ireland) was placed into an antecubital vein, and a resting blood sample (15 ml) was drawn. Immediately thereafter, subjects orally ingested antipyrine (10 mg/kg body mass; Janssen Chimica, Geel, Belgium). One hour after ingestion subjects cycled for 45 min at 50 % W_{max} (determined during the first cycling trial). Blood samples (10 ml) were drawn before and immediately after exercise. Blood was collected into

Table 1. Subject characteristics
(Mean values, standard errors and ranges)

	Supplemented group (*n* 11)*‡			Placebo group (*n* 9)†‡		
	Mean	SE	Range	Mean	SE	Range
Age (years)	60	1	55–66	59	1	55–65
Body mass (kg)	79·9	3·1	55·9–99·6	68·0	2·2	57·7–86·9
BMI (kg/m^2)	27	2	21–34	24	1	19–29
Body fat (%)	31	3	17–48	30	2	19–41
VO_{2max} (ml/min per kg FFM)	36	3	21–51	37	3	26–51
W_{max} (W)	146	19	70–270	139	13	85–230

VO_{2max}, maximal O_2 uptake; FFM, fat-free mass; W_{max}, maximal workload capacity.
* Five women and six men.
† Five women and four men.
‡ No significant differences were observed between the two groups (supplemented *v.* placebo).

EDTA- (1·34 mM) and GSH- (0·65 mM) containing tubes and was centrifuged immediately (3000 rpm, 10 min at 4°C). Aliquots of plasma were frozen in liquid N_2 and stored at −20°C until further analysis. Resting blood samples (5 ml) for determination of antioxidant status and cholesterol levels were collected into EDTA-containing tubes.

Supplementation

Subjects were either supplemented for 12 weeks with one capsule per d containing placebo (microcrystallin cellulose) or with a capsule containing 100 mg DL-α-tocopheryl acetate, 200 mg ascorbic acid and 2 mg β-carotene (Roche Vitamins Benelux, Deinze, Belgium). Dutch Biofarmaceutics BV (DBF, Helmond, The Netherlands) produced the capsules.

Oxidative stress

Antipyrine and thiobarbituric acid-reactive substances (TBARS) were used as markers for oxidative stress. Antipyrine and its hydroxylates were measured in plasma by reversed-phase HPLC–MS as described earlier (Coolen *et al.* 1999). Briefly, a reversed-phase Supersphere RP18 Endcapped column (LC-Packings, Amsterdam, The Netherlands) was attached to a LC system consisting of a LC-10AT pump (Shimadzu Ltd, Kyoto, Japan), and a Triathlon autosampler (Spark Holland, Emmen, The Netherlands). The HPLC was connected to an API-300 LC/MS/MS (Perkin Elmer Sciex Instruments, Thornhill, Canada), which operated in the multiple reaction mode with Turbo ionspray as interface. Sample pre-treatment consisted of C_{18} solid phase extraction (Sep-Pak® C18 Cartridges; Waters, Milford, MA, USA) in order to wash out salts and proteins. Cartridges were conditioned with methanol and H_2O, and 450 μl plasma were then inserted in the cartridge, followed by 2 ml 10 mM-ammonium acetate buffer. The cartridge was flushed with 1·5 ml methanol to elute the target components. Samples were evaporated to dryness under N_2 pressure and dissolved with 450 μl H_2O after which they stayed in an ultrasonic waterbath (30°C) for 30 min. Afterwards, samples were filtered by using Spartan 13/20 filters (Schleicher & Schuell, Dassel, Germany).

Since a competitive effect exists between antipyrine and other biomolecules for reaction with hydroxyl radicals, the formation of antipyrine hydroxylates is dependent on the available concentration of antipyrine. Therefore, phenolic derivatives: native antipyrine ratios are used, similar to the salicylic acid method (McCabe *et al.* 1997).

TBARS were measured in plasma using a fluorescent thiobarbituric acid assay. Thiobarbituric acid (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), 0·375 g, was dissolved in 250 ml H_2O and 2·5 ml 1 M-HCl. Plasma (111 μl) and TBA solution (1000 μl) were mixed and vortexed in an Eppendorf cup, which was then placed in a waterbath (95°C) for 1 h. Samples were cooled down to room temperature and the absorption was measured spectrophotometrically at 532 nm (Spectronic 1001; Meyvis, Bergen op Zoom, The Netherlands). Results were expressed as μmol malondialdehyde.

Antioxidant status

Antioxidant intake was derived from total food intake measured with a 7 d dietary record (Goris & Westerterp, 1999). Subjects received instructions from a dietitian on how to keep a food record and were asked not to change their habitual food intake. From the data of food records, antioxidant intake was calculated by a computer program based on food tables (Becel Nutritional Program, 1988; Nederlandse Unilever bedrijven BV, Rotterdam, The Netherlands). In addition, plasma was analysed for the α-tocopherol and β-carotene content by HPLC (Hess *et al.* 1991) on an Inertsil ODS-2, C_{18} reversed-phase column (GL Sciences Inc., Tokyo, Japan). Fluorescence detection was used for the determination of α-tocopherol. Simultaneously, β-carotene was detected by absorbance. Chromatogram peak areas were calculated with Gynkosoft Chromatography Data System (Gynkotek GmbH, Germering, Germany), and calibrated against a mixture of various standard substances dissolved in ethanol–dioxane–acetonitrile (1:1:3, by vol.). To adjust α-tocopherol levels for the total cholesterol concentration in plasma, total cholesterol was determined enzymatically (CHOD-PAP method; Monotest cholesterol, Boehringer, Mannheim, Germany) with a COBAS FARA semiautomatic analyser (Hoffman-La Roche, Basel, Switzerland).

Statistics

Data are presented as mean values with their standard errors. The non-parametric Mann-Whitney U-test was used to evaluate differences between groups, while the non-parametric Wilcoxon signed-rank test was used to evaluate differences within groups. Statistical significance was accepted as $P<0·05$. The StatView 5.0 program (SAS Institute Inc., Cary, NC) was used as the statistical package.

Results

Effect of antioxidant supplementation

Supplementation with antioxidant vitamins for 12 weeks resulted in a significant increase in the plasma concentrations of α-tocopherol (Δ 14·4 (SE 3·2) μmol/l, $P<0·001$) and β-carotene (Δ 0·4 (SE 0·1) μmol/l, $P<0·01$) in the supplemented group. No significant changes in plasma concentrations of α-tocopherol (Δ 5·6 (SE 2·1) μmol/l) or β-carotene (Δ 0·1 (SE 0·2) μmol/l) were observed in the placebo group. In the supplemented group, α-tocopherol levels in plasma were also significantly increased after adjusting for the plasma cholesterol level (Δ 2·5 (SE 0·5) mmol/mol, $P<0·001$). As a consequence of the supplementation, daily intake of ascorbic acid and β-carotene was significantly increased (Table 2).

Exercise-induced oxidative stress

In both groups, the 45 min cycling test resulted in significant ($P<0·0001$) increase in the plasma levels of TBARS (Δ 0·10 (SE 0·05) and Δ 0·07 (SE 0·03) μM for supplemented and placebo group respectively). In addition, the *p*- and *o*-APOH: native antipyrine ratios were significantly

Table 2. Dietary intake and plasma concentrations of antioxidants before and after 12 weeks supplementation with antioxidant vitaminst†
(Mean values, standard errors and ranges)

	Supplemented group‡							Placebo group						
	Baseline			12 weeks				Baseline			12 weeks			
	Mean	SE	Range	Mean	SE	Range	Change	Mean	SE	Range	Mean	SE	Range	Change	SE	Range
Dietary intake																
Energy (MJ/d)	9·5	0·8	5·9–13·2	9·2	0·6	5·5–11·4	−0·3	9·2	0·8	6·6–14·5	8·4	0·7	3·9–10·4	−0·8	0·6	−4·2–1·1
Ascorbic acid (mg/d)	74	13	16–171	273****	12	218–333	199	82	14	36–117	88	14	34–154	6	16	−83–56
β-Carotene (mg/d)	0·7	0·1	0·1–0·6	2·8****	0·1	2·3–3·6	2·1	0·8	0·1	0·4–1·5	0·8	0·1	0·1–1·3	0·0	0·1	−0·6–0·3
Plasma concentration																
α-Tocopherol (μmol/l)	25	1·7	17–33	39****	2·0	25–45	14	24	2·0	16–33	30	1·9	26–36	6	2·7	−2·3–15
α-Tocopherol:TC (mmol/l)	4·7	0·4	3·5–6·6	7·2***	0·3	4·9–9·1	2·5	4·3	0·3	3·2–60	5·2	0·3	4·6–6·0	0·9	0·5	−0·6–2·4
β-Carotene (μmol/l)	0·7	0·2	0·06–2·1	1·1**	0·2	0·17–2·1	0·4	1·1	0·2	0·2–1·7	1·0	0·2	0·2–1·7	−0·1	0·1	−0·9–0·2

TC, total cholesterol.
Mean values were significantly different from those at baseline: ** $P<0·01$, *** $P<0·001$, **** $P\leq0·0001$.
† For details of subjects and procedures, see Table 1 and p. 570.
‡ 100 mg DL-α-tocopheryl acetate, 200 mg ascorbic acid and 2 mg β-carotene/d (Roche Vitamins Benelux, Deinze, Belgium).

increased immediately after exercise (Table 3). The increase in the *p*- and *o*-APOH: antipyrine ratios in the supplemented group were $5·1\times10^{-4}$ (SE $2·0\times10^{-4}$) and $2·3\times10^{-4}$ (SE $0·9\times10^{-4}$) respectively ($P<0·0001$). The increase in the *p*- and *o*-APOH: antipyrine ratios in the placebo group were $3·3\times10^{-4}$ (SE $0·5\times10^{-4}$) and $2·2\times10^{-4}$ (SE $1·0\times10^{-4}$) respectively ($P<0·0001$).

Effect of antioxidant supplementation

Although supplementation for 12 weeks significantly increased the plasma antioxidant level, no effect on the exercise-induced increase in the *p*- and *o*- APOH: antipyrine ratios were observed (Table 3). In addition, no differences within and between both groups were observed in the exercise-induced increase of the plasma levels of TBARS before and after 12 weeks (Table 3).

Discussion

Recently, we showed in 60-year-old human subjects that a submaximal bout of exercise significantly increased the exercise-induced oxidative stress (Meijer *et al.* 2001), whereas no significant increase was observed in a group of young adults, who exercised at the same relative intensity (50 % W_{max}) (Coolen, 2000). Therefore, it was hypothesized that supplementation with antioxidants could possibly reduce the increase in exercise-induced oxidative stress in older adults. The results of the present study, however, showed that 12 weeks of antioxidant vitamin supplementation in 60-year-old subjects had no effect on the exercise-induced increase in oxidative stress, as measured by free radical reaction products of antipyrine. In addition, no change was observed in the exercise-induced increase of TBARS. It has to be mentioned, however, that the thiobarbituric acid assay lacks specificity when applied to human plasma.

The finding that antioxidant supplementation had no effect on exercise-induced oxidative stress is in accordance with previous studies that have relied on endogenous markers (Witt *et al.* 1992; Kanter *et al.* 1993; Maxwell *et al.* 1993). Witt *et al.* (1992) were the first to study the influence of a combined antioxidant supplement. They used urinary output of 8-hydroxyguanosine, a marker of RNA damage, as indicator for oxidative stress. Their subjects were moderately trained and cycled at 65 % maximal O_2 uptake for 90 min on three consecutive days. There was no evidence of exercise-induced damage in their control group, and subjects who took a combination of vitamin E, ascorbic acid and β-carotene for 1 month did not differ from controls. In addition, Maxwell *et al.* (1993) reported that in twenty-four younger adults (17–22 years), supplementation for 3 weeks with either placebo, 400 mg ascorbic acid or 400 mg DL-α-tocopheryl acetate did not affect the exercise-induced increase in the plasma level of TBARS. Furthermore, Kanter *et al.* (1993) concluded from their study in younger adults (20–29 years) that 6 weeks of combined ingestion of α-tocopherol, ascorbic acid and β-carotene did not prevent the exercise-induced increase in oxidative stress.

The only study that examined the effect of antioxidant supplementation on exercise-induced oxidative stress in

Table 3. Indicators for exercise-induced oxidative stress before and after 12 weeks supplementation with antioxidant vitamins†
(Mean values, standard errors and ranges)

	Supplemented group‡§						Placebo group					
	Baseline			12 weeks			Baseline			12 weeks		
	Mean	SE	Range	Mean	SE	Range	Mean	SE	Range	Mean	SE	Range
p-APOH:AP pre-exercise ($\times 10^{-4}$)	10·4	1·5	5·9–22·5	12·5	1·7	6·1–23·3	11·1	1·5	4·2–16·2	12·9	1·5	5·4–18·1
p-APOH:AP post-exercise ($\times 10^{-4}$)	15·6****	3·3	7·2–47·3	16·6****	2	9·5–29·8	14·5	1·7	6·2–17·7	17·1****	2·0	7·8–26·0
Change ($\times 10^{-4}$)	5·2	0·7	1·1–7·6	4·1	0·6	0·6–6·4	3·4	0·7	0·4–6·2	4·2	1·0	−0·2–9·2
Change:before (%)	50	10	10–99	33	7·0	1–94	31	6·0	3–64	33	7	−12–67
o-APOH:AP pre-exercise ($\times 10^{-4}$)	11·1	2·3	3·6–32·3	9·6	1·3	4·9–16·3	8·9	1·5	4·3–14·8	10·5	1·2	5·6–17·7
o-APOH:AP post-exercise ($\times 10^{-4}$)	13·4****	2·5	3·4–47·3	10·9****	1·4	5·2–17·6	11·4****	1·9	6·6–24·4	12·6****	1·5	6·9–21·6
Change ($\times 10^{-4}$)	2·3	0·9	0·5–11·2	1·3	0·9	−0·1–3·6	2·5	0·9	0·3–9·6	2·1	1·1	−0·5–9·4
Change:before (%)	21	14	0–36	14	3	−1–32	28	8	3–65	20	9	−3–78
TBARS pre-exercise (μM)	0·38	0·03	0·25–0·67	0·29	0·02	0·22–0·43	0·36	0·02	0·26–0·49	0·25	0·02	0·15–0·33
TBARS post-exercise (μM)	0·49****	0·07	0·27–1·11	0·44****	0·07	0·25–1·03	0·42****	0·05	0·27–0·76	0·35****	0·05	0·2–0·70
Change (μM)	0·11	0·04	0·01–0·44	0·15	0·06	−0·02–0·65	0·06	0·03	−0·05–0·28	0·10	0·04	−0·03–0·41
Change:before (%)	29	9	4–100	52	14	−8–82	16	7	−15–61	40	14	−10–62

p-APOH, *para*-hydroxyantipyrine; AP, antipyrine; *o*-APOH, *ortho*-hydroxyantipyrine; TBARS, thiobarbituric acid reactive substances.
Mean values were significantly different from those pre-exercise: **** $P<0·0001$.
† For details of subjects and procedures, see Table 1 and p. 570.
‡ 100 mg DL-α-tocopheryl acetate, 200 mg ascorbic acid and 2 mg β-carotene/d (Roche Vitamins Benelux, Deinze, Belgium).
§ There were no significant differences between and within the groups before and after 12 weeks of intervention.

older adults reported a significant reduction in urinary output of TBARS after 48 d α-tocopherol supplementation (Meydani *et al.* 1993). The older subjects (55–74 years), however, were supplemented with much higher doses of α-tocopherol (800 mg/d) when compared with the present study. Although subjects in the present study were supplemented for 12 weeks with relatively low doses of DL-α-tocopheryl acetate (100 mg), ascorbic acid (200 mg), and β-carotene (2 mg), supplementation significantly increased the plasma levels of α-tocopherol (Δ 14·4 (SE 3·2) μmol/l, $P<0·001$) and β-carotene (Δ 0·4 (SE 0·1) μmol/l, $P<0·01$). Ascorbic acid levels in plasma were not measured, but it might be assumed that those levels were also significantly increased after 12 weeks of supplementation, because there was a 3-fold increase in ascorbic acid intake (Table 2). It has been shown that the intake of 200 mg ascorbic acid saturates plasma almost 90 % (Levine *et al.* 1996). Therefore, the doses of antioxidant supplementation used in the present study should have been sufficient to increase the antioxidant status and capacity of exercising elderly.

It could be argued, however, that the number of subjects used in the present study was too low to detect any significant change of antioxidant supplementation on exercise-induced oxidative stress (*n* 11 and *n* 9 for the two groups). It has to be mentioned, however, that Meydani *et al.*(1993) used only twelve elderly subjects and showed that 48 d α-tocopherol supplementation (*n* 6) significantly decreased the urinary levels of TBARS after a bout of eccentric exercise. In addition, Hartmann *et al.* (1995) demonstrated in eight subjects that vitamin E supplementation prevented exercise-induced DNA damage. Therefore, the number of subjects used in the present study should have been sufficient to observe the potential for a decrease in exercise-induced oxidative stress after 12 weeks antioxidant supplementation. Another point that could be raised is that although there were no significant differences between the two groups, large inter-individual differences exist. Since the outcome measures on oxidative stress were independent of factors such as body mass and sex, the results have to be interpreted with some caution.

Although the protective role of α-tocopherol during exercise-induced oxidative stress is well established, it has to be mentioned, however, that the role of ascorbic acid is less clear (Ji, 1999). Ascorbic acid is known to play a role in sparing vitamin E, by regenerating α-tocopherol from the oxidized tocopheroxyl radical (Packer *et al.* 1979; Sies & Stahl, 1995). Furthermore, ascorbic acid is effective in preventing the oxidation of the blood GSH pool during physical exercise (Sastre *et al.* 1992). So far, only Ashton *et al.* (1999) showed that acute administration of ascorbic acid prevented exercise-induced oxidative stress during a maximal exercise test to exhaustion as measured by electron spin resonance spectroscopy (spin-trap α-phenyl-*tert*-butylnitrone). They suggested that ascorbic acid could be an effective antioxidant in the prevention of exercise-induced oxidative stress. It may be that human subjects only benefit from antioxidant supplements if they are deficient or exposed to exceptionally heavy workloads like maximal exercise tests or eccentric exercise protocols.

The exercise protocol used in the present study was not exceptionally high: subjects cycled for 45 min at an exercise intensity of 50 % W_{max} (about 55 % maximal O_2 uptake). However, the elderly human subjects in the present study were relatively unfit, and it has been shown that exhaustive exercise results in an increased oxidative stress (Powers *et al.* 1994; Ji, 1999). Indeed, we observed a significant increase in the free radical reaction products of antipyrine after exercise ($P<0{\cdot}0001$), similar to findings of a previous study in 60-year-old subjects (Meijer *et al.* 2001). The exercise-induced increase in the plasma levels of TBARS was also highly significant ($P<0{\cdot}0001$), which indicates that cycling for 45 min at 50 % W_{max} results in a significantly increased oxidative stress. Furthermore, it is known that older adults are more susceptible to oxidative stress compared with younger adults (Mecocci *et al.* 1999). In addition, ageing is associated with an increased mitochondrial free radical production (Shigenaga *et al.* 1994). Therefore, one might intuitively have suspected a decreased exercise-induced oxidative stress after 12 weeks of antioxidant supplementation.

Moreover, human subjects may benefit from antioxidant supplements if their antioxidant status is too low. Subjects in the present study, however, were not deficient concerning antioxidant intake. Measured vitamin C intake was higher than the Dutch recommended dietary allowance of 70 mg/d (Table 2). In addition, plasma concentrations of α-tocopherol and β-carotene were similar to findings of previous studies in elderly populations (Meydani *et al.* 1994; Jacques *et al.* 1995; Fotouhi *et al.* 1996; Pallast *et al.* 1999). It has been hypothesized that antioxidant supplementation is warranted in older subjects having a high daily physical activity level (Clarkson & Thompson, 2000; Polidori *et al.* 2000). Although the subjects in the present study were characterized as sedentary–active, further research on this topic is needed.

It could be argued that the method used (antipyrine oxidation) is not sensitive enough to measure the small changes after 12 weeks of antioxidant supplementation. However, the method used has been validated *in vitro* as well as *in vivo* conditions before. It was shown *in vitro* that exposure of an antipyrine solution in water to ^{60}Co γ-radiation leads to the formation of three phenolic antipyrine derivatives: *p*-, *o*-APOH and *meta*-hydroxyantipyrine (Coolen *et al.* 1997). When an antioxidant is added to an antipyrine solution in water, the conversion of antipyrine after exposure to ^{60}Co γ-radiation can be used as an indicator for the free radical scavenging capacity of a potential antioxidant (Coolen, 2000). For example, ascorbic acid significantly reduced the conversion of antipyrine, depending on the added concentration of ascorbic acid. The results of the antipyrine method were linearly correlated with the results of the commonly used deoxyribose assay (r^2 0·99). The properties of antipyrine make it also a suitable marker for measuring oxidative stress *in vivo*. A study in patients with claudicatio intermittens showed that 5 min walking exercise resulted in a significant increase in the plasma concentration of the free radical reaction products of antipyrine (Wijnen *et al.* 2001). Claudicatio intermittens is characterized by a chronic ischaemia–reperfusion injury during physical exercise, which results in an increased oxidative stress. Interestingly, the exercise-induced increase in the *o*-APOH: antipyrine ratio was similar in claudicants performing 5 min walking exercise ($\Delta 2{\cdot}1 \times 10^{-4}$) compared with older adults cycling for 45 min at submaximal intensity ($\Delta 2{\cdot}2 \times 10^{-4}$), which confirms that the increased hydroxyl radical formation in the exercising older subjects could be detected by the antipyrine method.

In conclusion, 12 weeks of antioxidant supplementation intervention in 60-year-old subjects had no effect on the exercise-induced increase in oxidative stress during 45 min cycling at submaximal intensity as measured by free radical products of antipyrine.

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