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/m2). 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 (D 14: 4( SE 3: 2) and 0: 4( SE 0: 1) μmol/l; P<0:001 and P<0:01). 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 tocopheryl 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\textsubscript{max}, maximal loading capacity.

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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 increase 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 60Co 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 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, Wmax and maximal O2 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 Cath6II; 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% Wmax (determined during the first cycling trial). Blood samples (10 ml) were drawn before and immediately after exercise. Blood was collected into sodium heparin to prevent the formation of oxyhemoglobin or methemoglobin, which can cause a falsely high measurement of O2 uptake.

### Table 1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Supplemented group (n 11)$\dagger$</th>
<th>Placebo group (n 9)$\ddagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td><strong>Body mass (kg)</strong></td>
<td>79.9</td>
<td>3.1</td>
</tr>
<tr>
<td><strong>BMI (kg/m2)</strong></td>
<td>27</td>
<td>2</td>
</tr>
<tr>
<td><strong>Body fat (%)</strong></td>
<td>31</td>
<td>3</td>
</tr>
<tr>
<td><strong>VO2max (ml/min per kg FFM)</strong></td>
<td>36</td>
<td>3</td>
</tr>
<tr>
<td><strong>Wmax (W)</strong></td>
<td>146</td>
<td>19</td>
</tr>
</tbody>
</table>

VO2max: maximal O2 uptake; FFM, fat-free mass; Wmax, maximal workload capacity.

$\dagger$ Five women and six men.

$\ddagger$ Five women and four men.

$\ddagger$ No significant differences were observed between the two groups (supplemented vs. placebo).
Exercise-induced oxidative stress

In both groups, the 45 min cycling test resulted in significant increase in the 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/ml, P<0-001). As a consequence of the supplementation, daily intake of ascorbic acid and β-carotene was significantly increased (Table 2).

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.

Effect of antioxidant supplementation

Supplementation with antioxidant vitamins for 12 weeks resulted in a significant increase in the plasma concentrations of α-tocopherol (Δ 4-4 (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/ml, P<0-001). As a consequence of the supplementation, daily intake of ascorbic acid and β-carotene was significantly increased (Table 2).

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, C18 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 semi-automatic analyser (Hoffman-La Roche, Basel, Switzerland).

Results

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Statistics

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Table 2. Dietary intake and plasma concentrations of antioxidants before and after 12 weeks supplementation with antioxidant vitamins†

(Mean values, standard errors and ranges)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>12 weeks</th>
<th>Change</th>
<th>Placebo group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>Energy (MJ/d)</td>
<td>9.5</td>
<td>0.8</td>
<td>5.9–13.2</td>
<td>9.2</td>
</tr>
<tr>
<td>Ascorbic acid (mg/d)</td>
<td>74</td>
<td>13</td>
<td>16–171</td>
<td>273***</td>
</tr>
<tr>
<td>β-Carotene (mg/d)</td>
<td>0.7</td>
<td>0.1</td>
<td>0.1–0.6</td>
<td>2.8***</td>
</tr>
<tr>
<td>α-Tocopherol (μmol/l)</td>
<td>25</td>
<td>1.7</td>
<td>17–33</td>
<td>39***</td>
</tr>
<tr>
<td>α-Tocopherol:TC (mol/l)</td>
<td>4.7</td>
<td>0.4</td>
<td>3.5–6.6</td>
<td>7.2***</td>
</tr>
<tr>
<td>β-Carotene (μmol/l)</td>
<td>0.7</td>
<td>0.2</td>
<td>0.06–2.1</td>
<td>1.1**</td>
</tr>
</tbody>
</table>

**A**p < 0.01, ***A**p < 0.001, ****A**p < 0.0001.
† For details of subjects and procedures, see Table 1 and p. 570.
† 100 mg dl-α-tocopherol acetate, 200 mg ascorbic acid and 2 mg β-carotene (Roche Vitamins Benelux, Deinze, Belgium).

Discussion

Effect of antioxidant supplementation

Although the plasma antioxidant level, exocellular output of 8-hydroxyguanosine, a marker of RNA damage, as measured by free radical reaction products of antipyrine. In addition, exercise-induced increase of the plasma levels of TBARS, however, showed that 12 weeks of antioxidant vitamin supplementation in 60-year-old subjects had no effect on the exercise-induced increase of oxidative stress, as measured by thiobarbituric acid assay lacks specificity when applied to human plasma. Their subjects were young adults, who exercised at the same relative intensity (50% VO\text{\textsubscript{2}}\text{max}) (Cooken, 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 no change was observed in the exercise-induced increase of oxidative stress, as measured by 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 (Meijer et al. 1993). Witt et al. (1992) and Kaner et al. (1993) concluded from their study in younger adults (17–22 years), supplementation for 12 weeks significantly increased the plasma antioxidant level, no effect on the exercise-induced oxidative stress. Their subjects were four younger adults (17–22 years), supplementation for 3 weeks significantly increased the plasma antioxidant level, no effect on the exercise-induced oxidative stress (Witt et al. 1993). The increase in antioxidant supplementation had no effect on the exercise-induced oxidative stress (Witt et al. 1992). Kaner et al. (1993) reported that in twenty young adults, who exercised at the same relative intensity (50% VO\text{\textsubscript{2}}\text{max}) (Cooken, 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 no change was observed in the exercise-induced increase of oxidative stress, as measured by thiobarbituric acid assay lacks specificity when applied to human plasma.

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Table 3. Indicators for exercise-induced oxidative stress before and after 12 weeks supplementation with antioxidant vitamins†

(Mean values, standard errors and ranges)

<table>
<thead>
<tr>
<th></th>
<th>Supplemented group‡§</th>
<th>Placebo group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>12 weeks</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td>p-APOH:AP pre-exercise (×10^{-4})</td>
<td>10.4 (1.5)</td>
<td>12.5 (1.7)</td>
</tr>
<tr>
<td>p-APOH:AP post-exercise (×10^{-4})</td>
<td>15.6 (3.3)</td>
<td>9.5 (2.9)</td>
</tr>
<tr>
<td>Change (×10^{-4})</td>
<td>5.2 (0.7)</td>
<td>11.7 (7.6)</td>
</tr>
<tr>
<td>p-APOH:AP pre-exercise (×10^{-4})</td>
<td>0.1 (0.2)</td>
<td>0.6 (0.4)</td>
</tr>
<tr>
<td>p-APOH:AP post-exercise (×10^{-4})</td>
<td>1.4 (0.7)</td>
<td>1.6 (1.0)</td>
</tr>
<tr>
<td>Change (×10^{-4})</td>
<td>0.2 (0.1)</td>
<td>0.7 (0.2)</td>
</tr>
<tr>
<td>p-APOH:AP pre-exercise (×10^{-4})</td>
<td>0.2 (0.1)</td>
<td>0.6 (0.4)</td>
</tr>
<tr>
<td>p-APOH:AP post-exercise (×10^{-4})</td>
<td>0.3 (0.1)</td>
<td>0.6 (0.4)</td>
</tr>
<tr>
<td>Change (×10^{-4})</td>
<td>0.1 (0.1)</td>
<td>0.5 (0.2)</td>
</tr>
<tr>
<td>p-APOH:AP pre-exercise (×10^{-4})</td>
<td>0.1 (0.2)</td>
<td>0.6 (0.4)</td>
</tr>
<tr>
<td>p-APOH:AP post-exercise (×10^{-4})</td>
<td>1.4 (0.7)</td>
<td>1.6 (1.0)</td>
</tr>
<tr>
<td>Change (×10^{-4})</td>
<td>1.2 (0.2)</td>
<td>0.8 (0.3)</td>
</tr>
</tbody>
</table>

† For details of subjects and procedures, see Table 1 and p. 570.
‡ There were no significant differences between or within the groups before and after 12 weeks of intervention.
§ There were no significant differences between and within the groups before and after 12 weeks of intervention.

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older adults reported a significant reduction in urinary 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% \( W_{\text{max}} \) (about 55% maximal \( \dot{O}_2 \) 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 (\( P<0.0001 \)), 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 (\( P<0.0001 \)), which indicates that cycling for 45 min at 50% \( W_{\text{max}} \) results in a significantly increased oxidative stress. Furthermore, it is known that older adults are more susceptible to oxidative stress compared with younger adults (Meccoci et al. 1999). In addition, ageing is associated with an increased mitochondrial free radical production (Shigena 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 \( \alpha \)-tocopherol and \( \beta \)-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 \( ^{60}\text{Co} \gamma \)-radiation leads to the formation of three phenolic antipyrine derivatives: \( p^-\), \( o^-\text{APOCH} \) and \( \text{meta}-\text{hydroxyantipyrine} \) (Coolen et al. 1997). When an antioxidant is added to an antipyrine solution in water, the conversion of antipyrine after exposure to \( ^{60}\text{Co} \gamma \)-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 \( o^-\text{APOCH} \) antipyrine ratio was similar in claudicants performing 5 min walking exercise (\( \Delta 2.1 \times 10^{-4} \)) compared with older adults cycling for 45 min at submaximal intensity (\( \Delta 2.2 \times 10^{-4} \)), 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.

Acknowledgements

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