Effect of vitamin supplementation on cytokine response and on muscle damage after strenuous exercise

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Effect of vitamin supplementation on cytokine response and on muscle damage after strenuous exercise. Am J Physiol Cell Physiol 280: C1570–C1575, 2001.—The present double-blinded, placebo-controlled study investigated whether antioxidant vitamin supplementation was able to modulate the cytokine and lymphocyte responses after strenuous eccentric exercise. Furthermore, muscle enzyme release was examined to see whether antioxidant treatment could reduce muscle damage. Twenty male recreational runners randomly received either antioxidants (500 mg of vitamin C and 400 mg of vitamin E) or placebo for 14 days before and 7 days after a 5% downhill 90-min treadmill run at 75% V˙O₂ max. Although the supplemented group differed significantly with regard to plasma vitamin concentration before and after exercise when compared with the placebo group, the two groups showed identical exercise-induced changes in cytokine, muscle enzyme, and lymphocyte subpopulations. The plasma level of interleukin (IL)-6 and IL-1 receptor antagonist increased 20- and 3-fold after exercise. The plasma level of creatine kinase was increased sixfold the day after exercise. The concentrations of CD4+ memory T cells, CD8+ memory and naive T cells, and natural killer cells increased at the end of exercise. The total lymphocyte concentration was below prevalues in the postexercise period. In conclusion, the present study does not support the idea that exercise-induced inflammatory responses are induced by free oxygen radicals.

cytokines; muscle damage; antioxidants; free oxygen radicals

STRENuous EXerCISE INDUCES remarkable changes in the concentrations and composition of lymphocyte subsets (20, 22, 29). During exercise, predominantly lymphocytes with an activated phenotype (CD45RO+) are recruited to the circulation (11). Furthermore, exercise induces increased levels of a number of cytokines, such as tumor necrosis factor-α (28), interleukin (IL)-1β (28), IL-6 (28), and IL-1 receptor antagonist (IL-1ra) (28), thereby mimicking the sequential release of cytokines after trauma (24). IL-6 is produced in larger amounts than any other cytokine in relation to exercise. Thus it increases up to 100-fold after a marathon run (25, 26). IL-6 induces production of the anti-inflammatory cytokine IL-1ra (29). It has been shown that IL-6 is produced locally in the working skeletal muscle (14, 26), but the mechanisms underlying the production of IL-6 are largely unknown. A previous study suggested that exercise-induced cytokine production was linked to muscle damage (2). However, recent studies did not confirm this (4, 14), and it is likely that IL-6 is produced as a consequence of muscle contractions and is involved in repair mechanisms in relation to muscle damage.

During exercise, whole body oxygen consumption increases up to 20-fold (1), with an even more dramatic muscular oxygen expenditure. A rise in oxygen consumption will enhance the electron transfer through the respiratory chain and thereby increase free oxygen radical production, since 1–3% of the total oxygen consumed is transformed to free radicals (12). This could challenge the natural antioxidant defense, supposing that the body was lacking antioxidants during prolonged strenuous exercise, the cause being either antioxidant deprivation or a higher free oxygen radical to antioxidant ratio.

Free radicals are produced continuously by incomplete reduction of oxygen in the respiratory chain and from many cells, including leukocyte subsets (phagocytes and lymphocytes). Because of a high reactivity caused by unpaired electrons, free radicals can cause extensive damage to cells, including their membranes, DNA, and intracellular protein and carbohydrate structures (8). The cell damage or death caused by free oxygen radicals will thereby activate the cellular immune system, including inflammatory responses. Antioxidants abolish or minimize this reactivity by being reductants and include the lipophilic vitamin E and hydrophilic vitamin C (8).

Free oxygen radicals have been shown to increase the expression of some cytokines (10, 17). It is, there-

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fore, possible that antioxidant treatment would down-regulate the production of cytokines in exercise. Furthermore, aging is associated with increased levels of free oxygen radicals (23, 27), and elderly subjects who receive antioxidant vitamins have been shown to have an improved cellular immunity (5, 6, 19, 30). It is, therefore, possible that the provision of antioxidants would protect against exercise-induced immune activation.

We therefore hypothesized that the generation of free oxygen radicals in response to exercise might induce lymphocyte activation and increased production of cytokines. The latter might be due to a direct effect on the transcription rate (10). Alternatively, exercise-induced production of free oxygen radicals causing muscle damage could lead to activation of repair mechanisms involving production of cytokines such as IL-6.

To investigate this hypothesis, subjects were randomly separated into two groups receiving either antioxidants (vitamin C and vitamin E) or placebo before and after performing an exercise test. Furthermore, to investigate the proposed link between exercise-induced muscle damage and cytokine production (2), we used eccentric exercise as a model of muscle-damaging exercise. Thus 20 male volunteers performed 90 min of downhill treadmill running at a pace adjusted to 75% maximal oxygen consumpiton (Vo2 max).

**METHODS**

Twenty male subjects participated in the present randomized, double-blind, placebo-controlled study. Personal characteristics did not differ between the two groups (Table 1). After the purposes and risks of the protocol were explained, oral and written informed consent was obtained. The protocol

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**Table 1. Characteristics of subjects**

<table>
<thead>
<tr>
<th></th>
<th>Age, yr</th>
<th>Weight, kg</th>
<th>Height, cm</th>
<th>BMI, kg m−2</th>
<th>Vo2max, Vmin</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplemented</td>
<td>28(23–39)</td>
<td>77.7(67–92)</td>
<td>189(183–195)</td>
<td>22.6(18.4–24.5)</td>
<td>5.0(4.2–5.5)</td>
<td>10</td>
</tr>
<tr>
<td>Placebo</td>
<td>26(20–32)</td>
<td>75.7(54–85)</td>
<td>182(164–198)</td>
<td>22.1(20.7–23.7)</td>
<td>4.8(3.7–5.6)</td>
<td>10</td>
</tr>
</tbody>
</table>

Data are shown as means with range in parentheses. BMI, body mass index.

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**Table 2. Lymphocyte subpopulations of supplemented and placebo groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Start</th>
<th>Finish</th>
<th>1 h. post</th>
<th>2 h. post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>S 2.4 ± 0.2</td>
<td>4.7 ± 0.5***</td>
<td>6.3 ± 0.6***</td>
<td>9.0 ± 0.6***</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>S 1.8 ± 0.2</td>
<td>3.5 ± 0.4***</td>
<td>1.4 ± 0.1***</td>
<td>1.3 ± 0.1***</td>
</tr>
<tr>
<td>Monocytes</td>
<td>S 0.44 ± 0.03</td>
<td>0.58 ± 0.09**</td>
<td>0.53 ± 0.09</td>
<td>0.81 ± 0.12***</td>
</tr>
<tr>
<td>CD3+</td>
<td>S 0.97(0.84–1.26)</td>
<td>1.54(1.24–2.02)***</td>
<td>0.81(0.59–1.00)</td>
<td>0.76(0.56–0.96)***</td>
</tr>
<tr>
<td>CD4+</td>
<td>S 0.79(0.56–1.00)</td>
<td>1.25(1.06–1.42)***</td>
<td>0.79(0.57–1.02)</td>
<td>0.57(0.42–0.92)***</td>
</tr>
<tr>
<td>CD8+</td>
<td>S 0.53(0.49–0.73)</td>
<td>0.79(0.61–1.14)***</td>
<td>0.44(0.35–0.68)</td>
<td>0.43(0.28–0.62)***</td>
</tr>
<tr>
<td>CD4+CD45RA+CD62L+</td>
<td>S 0.01(0.01–0.04)</td>
<td>0.04(0.01–0.05)***</td>
<td>0.01(0.01–0.02)</td>
<td>0.01(0.01–0.02)</td>
</tr>
<tr>
<td>CD4+CD45RA+CD62L+</td>
<td>S 0.15(0.06–0.20)</td>
<td>0.20(0.03–0.11) ***</td>
<td>0.09(0.08–0.22)</td>
<td>0.10(0.07–0.14)</td>
</tr>
<tr>
<td>CD8+CD45RA+CD62L+</td>
<td>S 0.04(0.02–0.07)</td>
<td>0.08(0.03–0.11) ***</td>
<td>0.05(0.02–0.06)</td>
<td>0.04(0.02–0.06)</td>
</tr>
<tr>
<td>CD4+CD45RA+CD62L+</td>
<td>S 0.18(0.03–0.31)</td>
<td>0.15(0.02–0.40)***</td>
<td>0.10(0.01–0.24)</td>
<td>0.07(0.02–0.20)</td>
</tr>
<tr>
<td>CD3+CD45RA+CD62L+</td>
<td>S 0.14(0.01–0.22)</td>
<td>0.27(0.20–0.37) ***</td>
<td>0.14(0.11–0.39)</td>
<td>0.12(0.08–0.23)</td>
</tr>
<tr>
<td>CD3+CD45RA+CD62L+</td>
<td>S 0.08(0.04–0.14)</td>
<td>0.17(0.12–0.25)</td>
<td>0.10(0.04–0.18)</td>
<td>0.11(0.03–0.17)</td>
</tr>
<tr>
<td>CD8+CD45RA+CD62L+</td>
<td>S 0.06(0.02–0.17)</td>
<td>0.04(0.01–0.07)</td>
<td>0.02(0.01–0.05)</td>
<td>0.02(0.01–0.03)</td>
</tr>
<tr>
<td>CD3+CD45RA+CD62L+</td>
<td>S 0.05(0.04–0.12)</td>
<td>0.15(0.10–0.20)***</td>
<td>0.02(0.02–0.03)***</td>
<td>0.03(0.03–0.04)***</td>
</tr>
<tr>
<td>CD3+CD45RA+CD62L+</td>
<td>S 0.04(0.03–0.05)</td>
<td>0.07(0.07–0.09)***</td>
<td>0.02(0.03–0.05)***</td>
<td>0.02(0.01–0.04)***</td>
</tr>
<tr>
<td>CD3+CD45RA+CD62L+</td>
<td>S 0.01(0.01–0.04)</td>
<td>0.02(0.01–0.03)***</td>
<td>0.01(0.00–0.01)*</td>
<td>0.01(0.00–0.02)***</td>
</tr>
<tr>
<td>CD3+CD45RA+CD62L+</td>
<td>S 0.17(0.09–0.21)</td>
<td>0.26(0.14–0.40)***</td>
<td>0.11(0.03–0.19)</td>
<td>0.11(0.05–0.17)***</td>
</tr>
<tr>
<td>CD8+CD45RA+CD62L+</td>
<td>S 0.11(0.07–0.15)</td>
<td>0.16(0.12–0.25)***</td>
<td>0.12(0.06–0.19)</td>
<td>0.09(0.05–0.09)***</td>
</tr>
<tr>
<td>CD3+CD16+CD56+</td>
<td>S 0.02(0.02–0.08)</td>
<td>0.08(0.07–0.20)***</td>
<td>0.04(0.03–0.10)</td>
<td>0.03(0.01–0.11)***</td>
</tr>
<tr>
<td>CD3+CD16+CD56+</td>
<td>S 0.02(0.01–0.06)</td>
<td>0.02(0.02–0.11)***</td>
<td>0.01(0.01–0.03)</td>
<td>0.01(0.01–0.04)***</td>
</tr>
<tr>
<td>CD3+CD16+CD56+</td>
<td>S 0.01(0.01–0.02)</td>
<td>0.06(0.05–0.08)***</td>
<td>0.01(0.01–0.03)</td>
<td>0.01(0.00–0.01)***</td>
</tr>
<tr>
<td>CD3+CD16+CD56+</td>
<td>S 0.13(0.10–0.15)</td>
<td>0.58(0.34–0.77)***</td>
<td>0.03(0.01–0.04)***</td>
<td>0.03(0.03–0.04)***</td>
</tr>
<tr>
<td>CD3+CD16+CD56+</td>
<td>S 0.02(0.02–0.03)</td>
<td>0.05(0.04–0.07)***</td>
<td>0.01(0.01–0.01)</td>
<td>0.01(0.01–0.02)***</td>
</tr>
</tbody>
</table>

Lymphocyte subpopulation values are medians (25%–75% percentile). Leucocyte values are means ± SE. Concentrations are expressed as 10^6 cells/L. *Significantly different from start; P ≤ 0.05; **significantly different from start; P ≤ 0.01; ***significantly different from start; P ≤ 0.001. S, supplemented, P, placebo.
was approved by the local ethics committee of the Copenhagen and Frederiksberg community.

Test subjects were recruited on the basis of being accustomed to recreational training and not taking any medication or dietary supplementation. Furthermore, any participation in heavy eccentric-biased work 3 mo before, during, or 1 wk after the experiment excluded the subjects. The subjects were given a training diary to record any physical activity along with intensity during the trial period, thus ensuring that only light to moderate exercise was performed.

Supplementation. The subjects were randomly and evenly divided into a supplemented (S) group and a placebo (P) group. The supplementation consisted of capsules containing 500 mg of vitamin C and 400 mg of vitamin E, whereas the placebo consisted of similar capsules containing 900 mg of maltodextrin (both provided by Nestlé Research Center, Lausanne, Switzerland). Capsules were administered orally and daily for 2 wk before the experimental day and continued 1 wk after.

Exercise protocol. The exercise protocol consisted of a 5% downhill treadmill run for 1.5 h (Technogym, Italy). The pace was adjusted according to a workload of 75% of the individual maximal oxygen consumption ($75\% \text{ VO}_2\text{max}$). Furthermore, an oxygen consumption and pulse test was conducted 1 wk before the actual run. The same treadmill, heart rate recording (Polar advantage NV system; Kempele, Finland), and oxygen equipment (MedGraphics CPP-S and CPX; St. Paul, MN) were used in both trials.

During the actual run, heart rate was recorded continuously while oxygen consumption was monitored every 20 min, ensuring the proper workload. At 30 min and at 1 h into the run, the subjects were allowed a 2-min reduction of workload to 50% $\text{VO}_2\text{max}$, ensuring an easy water intake. Experimental day. The subjects reported to the laboratory in the morning after an overnight fast. During the entire trial period, water was allowed ad libitum. Half an hour of rest, the preexercise samples (start) were obtained. Immediately after the run (finish), and 1 and 2 h and 1, 2, and 7 days after, blood was sampled from an antecubital vein. All subsequent samples were obtained at the same time of day.

After the run, the subjects remained completely sedentary. The participants were asked to refrain from any strenuous exercise during the week after the trial day.

Cytokines. Blood was collected in prechilled tubes containing EDTA and Trasylol (Bayer, Leverkusen, Germany; 175 KIU (ml of aprotinin)$^{-1}$, where a KIU is a kallikrein inacti-
VITAMIN SUPPLEMENTATION, EXERCISE, AND IMMUNE PARAMETERS

C1573

vator unit, equivalent to 0.14 μg of crystalline aprotinin] for cytokine analysis. The samples were centrifuged cold (4°C) at 2,150 g for 15 min, and the plasma was subsequently frozen at −80°C for later analysis.

Cytokine analysis was carried out with commercially available (R&D Systems, Minneapolis, MN) enzyme-linked immunosorbent assay kits.

Vitamin E. After precipitation of the proteins in the sample by adding ethanol containing the internal standard, the vitamins were extracted with n-hexane. In separating the vitamins, an HPLC system was used containing a Suplex pKb precolumn and two Suplex pKb 100 columns (5 μm, 250-4.6 mm), gradient elution, diode-array detection, and fluorescence detector (the latter was used for tocopherol detection, excitation at 295 nm, emission at 320 nm). For further details, see Traber et al. (32).

Vitamin C. Immediately after separation, plasma was mixed with 10% metaphosphoric acid (1:1; vol:vol) and then stored at −80°C for 1 mo maximum. Samples were thawed and centrifuged at 3,000 g for 10 min and then injected on the HPLC system. The HPLC system consisted of an RP-C18 Novak-Pak column (Waters) protected by an ODS Hypersil (C18) column (Hewlett-Packard). The mobile phase was a potassium dihydrogenophosphate solution (5 g/l water) adjusted at pH 2.5 with orthophosphoric acid. The HPLC was run under isocratic conditions at 32°C. The vitamin C was detected using an ultraviolet detector and quantified using an external standardization.

Clinical chemistry tests. All tests involving measurement of muscle enzymes, leucocyte subsets, erythrocytes, and hemoglobin concentrations were carried out by the Department of Clinical Chemistry, Rigshospitalet, Copenhagen, Denmark.

All blood samples, except those collected for muscle enzyme analysis, were collected in tubes containing EDTA and processed with a cell counter (Technicon H. I. Miles, Tarrytown, NY). Lithium-heparinized plasma was used to analyze muscle enzymes using automated enzyme reactions (Hitachi System 717 and Boehringer Mannheim Diagnostica, Germany).

Isolation of blood mononuclear cells. Blood mononuclear cells were extracted from heparinized blood using density gradient centrifugation (Lymphoprep; Nyegaard, Oslo, Norway) on Leucosep tubes (Greiner, Frickenhausen, Germany). After isolation, cells were washed three times in RPMI 1640 (GIBCO, Grand Island, NY).

Flow cytometry analysis. The following anti-human antibodies were used to detect surface cell markers. Fluorescein isothiocyanate-conjugated antibodies, including mouse IgG1, mouse IgG2a, CD4 (clone T29/33), and CD45RO (clone MY31) and CD62L (clone SK11) (both from Becton Dickinson). Finally, CD3 (clone SK7), CD4 (clone SK3), and CD8 (clone SK1) were conjugated with peridinin chlorophyll protein, all supplied from Becton Dickinson. Mouse IgG1 and IgG2a served as controls.

Tubes containing antibody were each filled with 10^6 cells suspended in 100 μl of phosphate-buffered saline (PBS; J. T. Baker, Deventer, Holland) with 3% fetal calf serum (GIBCO). The samples were incubated for 30 min at 4°C and then washed twice in the solution described above. After being resuspended in 300 μl of PBS, the samples were subsequently processed by flow cytometry using a fluorescence-activated cell sorter (Epics XL-MCL; Coulter, FL). Data was analyzed using PC Lysis software from Becton Dickinson. All concentrations of listed lymphocyte subpopulations (Table 2) were ascertained by multiplying each percentage with the total lymphocyte cell count.

Statistical analysis. All data were corrected for dehydration according to the method described by Dill and Costill (9). The data were log transformed if a normal distribution did not occur. Data were analyzed for time and group intervariability using repeated-measures analysis of variance. Where no group effect was found, data was pooled. If the data showed significance, a t-test with Bonferroni correction was performed determining time points of significance. The following parameters were log transformed before statistical analyses: IL-6, IL-1ra, creatine kinase (CK), vitamin C, and vitamin E.

RESULTS

Effect of supplementation with vitamin C and vitamin E. The plasma concentration of both vitamins was increased throughout the entire trial in the supplemented group. A significant difference in plasma concentration of vitamins C and E was found separating the two groups (P < 0.001 and P < 0.000006, respectively). Vitamins C and E blood plasma levels rose 60 and 80%, respectively, in the supplemented group when day 14 values were compared with subsequent samples. Furthermore, the plasma concentration of vitamin C was transiently enhanced during exercise in both groups and remained elevated for at least 2 h (Fig. 1, A and B).

Cytokines. Plasma concentrations of IL-6 and IL-1ra increased 20- and 3-fold, respectively, following exercise compared with preexercise values. IL-6 peaked immediately after exercise, whereas IL-1ra peaked with a 2-h time delay. Both cytokines had returned to baseline values the day after exercise (Fig. 2, A and B).

CK. CK peaked on day 1 after the run with a 6-fold increase, subsequently displaying a slow decrease in the following days. CK values were still 2-fold elevated 7 days after the experimental day (Fig. 3).

Fig. 3. Plasma creatinine kinase (CK) concentrations are shown as geometric means with a 95% confidence interval in relation to 90 min of downhill treadmill exercise at a pace adjusted to 75% V̇O₂max. Blood samples were obtained 14 days, 7 days, and just before exercise (start), immediately after exercise (finish), and at 1 and 2 h and 1, 2, and 7 days after exercise. **Significantly different from start, P < 0.01; ***significantly different from start, P < 0.001.
Leukocyte subpopulations. The concentration of neutrophils increased twofold during exercise and continued to increase until an almost fourfold rise was observed 2 h later (Table 2). The lymphocyte count increased 2-fold during exercise, followed by a 20% decrease 2 h after exercise, compared with baseline values. Monocyte concentration rose 30% during exercise and continued to rise after exercise until a 75% increase was observed 2 h postexercise. All leukocyte concentrations had returned to baseline levels day 1 after exercise.

In general, the subpopulations followed the total lymphocyte trend (Table 2). CD3+ T cells showed a significant rise after exercise, before declining below preexercise levels 2 h later. CD4+ and CD8+ T cells showed similar patterns, except that a significant decrease was not found in both subpopulations until 2 h after the exercise.

The CD4+ cells expressing memory surface markers (CD45RO+) were likewise elevated after exercise, yet they showed no significant suppression. The CD8+ memory cells displayed exercise-induced elevations together with a significant decrease in the sedentary period. Where CD4+ true naïve cells (CD45RA−CD62L−) displayed no significance when tested against time, CD8+ true naïve cells showed a significant increase after exercise. Both CD4+ and CD8+ revertant cells (CD45RA−CD62L−) increased significantly during exercise. CD4+ revertant cells returned to baseline values, whereas CD8+ revertant cells dropped below baseline values 2 h postexercise.

Natural killer cell subpopulations, CD3+CD16+CD56−, CD3−CD16+CD56+, and CD3−CD16−CD56−, all peaked significantly during exercise and showed likewise decreases below baseline values 2 h postexercise (except for CD3−CD16−CD56+, which did not show any postexercise decrease).

The supplemented group displayed no significant differences compared with the placebo group with regard to all of the above-mentioned parameters, except for plasma vitamin levels. Thus the results represent pooled data from both groups.

DISCUSSION

The main purpose of the study was to evaluate whether antioxidant vitamin supplementation had any effect on the cytokine, muscle enzyme, or lymphocyte responses to exercise. In line with previous studies (29), we demonstrated significant increases in IL-6, IL-1ra, and CK after eccentric exercise. The present study also confirmed previous findings showing that all lymphocyte subpopulations are recruited to the blood during exercise and that the total lymphocyte count is suppressed in the postexercise period (22, 29). Analyses of blood demonstrated that we were able to identify two groups (supplemented and placebo) that differed markedly with respect to the vitamins C and E concentrations in the blood. However, the two groups had the same cytokine and lymphocyte responses to exercise. The latter findings are in agreement with those of Nieman et al. (21) who found that 8 days of vitamin C supplementation (1,000 mg/day, n = 6) had no influence on immune parameters after a 2.5-h run compared with a control group (n = 6).

In this study, we also evaluated the effect of antioxidant supplementation on exercise-induced muscle damage. However, vitamin supplementation did not influence exercise-induced muscle damage as visualized by CK levels. This finding is in agreement with studies by Warren et al. (33) and Kaikkonen et al. (15) but differ from McBride et al. (18) and Rokitzki et al. (31). The discrepancies could be a result of varied exercise protocols and different duration, quantities, and types of antioxidant supplementation used during the trials. Furthermore, we did not find any correlation between cytokine production and CK. Thus the present study does not support the idea that exercise-induced cytokine production is linked to muscle damage.

The body possesses a natural selection of antioxidant enzymes and coenzymes, which minimizes free oxygen radical damage (7, 13). During periods of increased oxidative stress, e.g., during exercise, an enhancement of these natural antioxidants could occur. In both groups, we observed an increase in the plasma concentration of vitamin C during and after exercise, which could represent a reserve released during periods of greater oxidative stress. Perhaps this could sustain the free oxygen radical-to-antioxidant ratio, thereby eliminating excessive cellular damage. Elite athletes with a high-training regimen have been shown to have elevated baseline antioxidant levels compared with sedentary subjects (3, 16), suggesting an adaptive mechanism. The natural increase in plasma vitamin concentration during and after exercise could possibly abolish the effect of additional vitamin supplementation, though this needs to be further explored.

In conclusion, the present study showed that supplementation with vitamin C and vitamin E combined significantly enhanced the levels of these vitamins in the blood without influencing the cytokine, muscle enzyme, or lymphocyte responses to exercise. Thus this study does not support the idea that the exercise-induced immune changes are generated by free oxygen radicals.

The excellent technical assistance of Ruth Rousing, Hanne Willemmsen, and Birgit Mollerup is acknowledged. This project was supported by Nestlé Research Center.

REFERENCES