A simple test to monitor oxidative stress

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Background. The role of oxygen free radicals is considered important in the development of cardiovascular disease. However, until recently determination of free radicals plasma levels and the effect of antioxidant therapy on these levels has been difficult. The aim of the study was to determine the oxidative stress and the effect of the antioxidant compound ARB, Stenovit on this stress in normal subjects and patients with intermittent claudication after oral administration for one week.

Methods. A portable, free radicals (FRs) determination system (D-Roms test, Diascor, Grosseto, Italy) was used. This test is based on the ability of transition metals to catalyse in the presence of peroxides with formation of FRs which are trapped by an alchilamine. The alchilamine reacts forming a coloured radical detectable at 505 nm. The reagents utilised are the cromogen (R1, an alchilamine) and a pH 4.8 buffer (R2). Ten µl of hemolysis-free serum are to 1 ml of R2 and to 10 µl of R1. The sample is mixed, incubated (1 mini; 37°C) and read for optical density. After another minute, the sample is read again. The average delta 4mn is multiplied by a K factor calculated using serum with defined value.

Results. In normal subjects the mean (sd) levels of free radicals were 3.12±1.49 U.CARR (Carratelli units) before treatment and 21.4±3.3 U.CARR after treatment (p<0.05). A decrease of at least 10% was detected in every subject. In patients with peripheral vascular disease the mean (sd) levels of free radicals were 40.1±4.2 U.CARR before treatment and 27.1±3.3 U.CARR after treatment (p<0.02). A decrease of at least 15% was detected in every patient (median value 31%).

Conclusions. The D-Roms test provides a simple, inexpensive and practical method to identify subjects with a high level of oxidative stress and to demonstrate the effect of treatment. The compound ARB, Stenovit is effective in reducing circulating free radicals. Its action on the progression of atherosclerotic disease should be assessed in future studies.

Keywords: Free radicals - Antioxidants - Vascular disease - Oxidative stress.

The relationship between oxidative stress, plasma lipoprotein levels, other cardiovascular risk factors and the development of atherosclerotic cardiovascular disease is considered important.1 Oxidative stress appears to be associated with an increased production of oxygen free radicals which alters the natural antioxidant defence mechanisms present in most tissues.2-5 Dietary changes such as reduction in fat intake, increase in fruit and vegetable intake, and vitamin E supplements delay the development of symptomatic cardiovascular disease.6-10 Long term studies have produced conflicting results after prolonged administration of antioxidants.11,12 Reactive oxygen may cause important cell damage by the oxidation of membranes or by altering critical enzyme pathways and systems.3 Among the several free-radicals, the hydroxyl (•OH) which is responsible for the oxidation of many organic substances such as proteins and particularly lipids is considered to be the most powerful.2 Lipid oxidation is essential for the progression of atherosclerotic plaques.13,14 Free radicals are extremely reactive and have a very short half life. They may be measured directly using the spin trapping method.15 Free radicals react with a trap molecule which produces a more stable product detectable by electron spin resonance. Reproducibility problems have not been overcome despite the use of different trap molecules (dimethylsulphoxide, salicylate, phenylethylamine, iensphenetic acid).16,17 More complex methods have been developed18 but they are not practical for routine clinical applications or screening.

Recently a simple method has been developed, the D-Roms test.19 This method is based on the ability of transition metals to catalyse in the pres-
ence of peroxides, the formation of free-radicals which are then trapped by an alchylamine. The alchylamine reacts forming a coloured radical detectable at 505 nm through a kinetic reaction which is linear up to 500 U.CARR (Carraelli Units). The determination of free-radicals can be made with a normal spectrophotometer. The normal range has been determined as 250-300 U.CARR.21

The aim of the study was to determine the plasma changes in free-radicals produced by a new compound (AR10 Stenovit) using the D-Roms test in normal subjects and patients with peripheral vascular disease.

Materials and methods

The D-Roms Test

The reagents utilised are the chromogen R1 (an alchylamine) and R2 a pH 4.8 buffer. Ten µl of haemolysis free serum is added to 1 ml of R2 and the mixture is immediately added to 10 µl of R1. The sample is gently mixed and incubated in a cuvette for 1 minute at 37°C. It is then read for optical density at 505 nm using a spectrophotometer. After another minute, the sample is read again, the difference (delta A) is determined and multiplied by a K factor (9000). Thus the result is calculated according to the formula:

delta A × K = U.CARR

The antioxidant compound

This was provided in 10 ml of two phase vials administered orally once per day. The ingredients consisted of membrane antioxidants (vitamins E, A, and D-carotene), circulating antioxidants (vitamin C, flavonoids and Coenzyme Q10), antioxidants which are part of physiological antioxidant systems such as GSH and POD (L-cysteine, zinc and selenium) and vitamin B6 which is required for the metabolism of homocysteine. The amounts of each ingredient is shown in Table 1.

Subjects

Twelve normal subjects (mean age 56±13), 6 males and 6 female, were admitted to the study. None of them smoked and ultrasonic scan demonstrated that the carotid and common femoral bifurcations were normal. Fifteen patients with intermittent claudication (200-500 m), (mean age 65±13), 9 male and 6 female, were also admitted to the study. They had absent or reduced pulses. Their walking distance was documented on a treadmill at 3 km/h at an inclination of 10%. They were all taking aspirin 300 mg daily. Diabetic, hypertensive and hyperlipidaemic patients were excluded. All patients had given up smoking at least 4 weeks before inclusion into the study.

Procedure

The compound was administered orally before breakfast for one week. Blood samples (0.2 ml) were taken from fingertips at 8 am the day before administration of the antioxidant compound started (day 1) and on the first day after the treatment period (day 9). The blood samples were immediately tested with the D-Roms test.

Statistical analysis

Because the coefficient of variation of the D-Roms test is less than 5% the number of the subjects selected acting as their own controls was considered sufficient to detect a change produced by the treatment. The paired "t"-test was used.

Results

In normal subjects the mean (±SD) levels of free radicals 312±49 U.CARR before treatment and 218±33 U.CARR after treatment (p<0.05). A decrease of at least 10% was detected in every subject. In patients with peripheral vascular disease the mean (±SD) levels of free radicals were 404±42 U.CARR, before treatment and 278±33 U.CARR after treatment (p<0.02). A decrease of at least

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**Table 1.—Ingredients of AR10 Stenovit.**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E (mg)</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin A (mg)</td>
<td>0.4</td>
</tr>
<tr>
<td>D-carotene (mg)</td>
<td>0.05</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>30</td>
</tr>
<tr>
<td>Coenzyme Q10 (mg)</td>
<td>20</td>
</tr>
<tr>
<td>Flavonoids (from citrus)</td>
<td>30</td>
</tr>
<tr>
<td>L-Cysteine (mg)</td>
<td>10</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>5</td>
</tr>
<tr>
<td>Selenium (μg)</td>
<td>48</td>
</tr>
<tr>
<td>Vitamin B6 (mg)</td>
<td>1</td>
</tr>
</tbody>
</table>
15% was detected in every patient (median value 31%).

**Discussion and conditions**

Oxidative stress appears to be associated with an increase in production of free radicals altering the natural antioxidant defence mechanisms present in most tissues. It has been observed that dietary changes such as a reduction in fat intake, increased fruit and vegetable intake and vitamin A supplements may prevent or delay the development of symptomatic cardiovascular disease. Long term studies have observed conflicting results after prolonged administration of beta-carotene and other antioxidants.

Theoretically the protective action of antioxidants may be obtained by natural dietary elements rather than by administration of pharmacological supplements. Every antioxidant is a redox agent which protects against free radicals but in some circumstances promotes production of the latter. When antioxidants are given in physiological concentrations according to the Recommended Dietary Allowance (RDA) they are present in both the reduced and oxidised state. In pharmacological supplements antioxidants are present only in the reduced state and paradoxically may increase the generation of free radicals promoting or accelerating the evolution of cardiovascular, liver and kidney disease and even cancer.

According to the above consideration oxidative stress appears to be clinically relevant. However, so far no large studies have been conducted in which oxidative stress changes were correlated to the clinical outcome. The D-Roms test validated in the clinical setting is a simple and reliable test to determine the level of oxidative stress. In patients with symptomatic coronary artery disease values greater than 400 U.CARR are detected in 75% of cases. The D-Roms test can be used to study and monitor the effects of antioxidants and has the potential to identify responders and non-responders to treatment. Further research is needed to evaluate the role of antioxidant compounds in diseases associated with an increased oxidative stress.

A complex compound which includes several antioxidant elements may be more effective than the administration of individual antioxidants.

An appropriate diet containing all the elements used in the ARчетный Stenovit is theoretically possible but difficult to achieve as the quantity of antioxidants in the diet tends to be variable and largely unpredictable. The possibility of measuring oxidative stress *in vivo* with a simple, inexpensive and reliable test means that it is now possible (a) to identify subjects with a high level of oxidative stress; (b) to monitor efficacy in terms of reduction of oxidative stress and (c) to correlate changes in oxidative stress in relation to the clinical efficacy of treatment in terms of clinical outcome.

**References**


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