
Evaluating an individual's oxidative stress: a reality for doctors

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"Not all individuals have the same level of oxidative stress. By determining a person's antioxidant profile as early as possible in life, it is possible to take precautions so as to delay many age-related diseases."

R Cutler, Genox Corporation, Baltimore, USA

Introduction

Oxidative stress is defined as an imbalance between pro- and antioxidants, leading to irreversible cell damage. Reduction of monovalent oxygen results in the formation of activated oxygen species (AOS) including free radicals (superoxide anion, hydroxyl radical), hydrogen peroxide, and singlet oxygen. All of these species are potentially toxic to the organism because they can inactivate proteins, induce breaks in deoxyribonucleic acid (DNA) and thus alter the genetic message, degrade sugars, oxidise lipoproteins, and initiate lipid peroxidation in cell membranes by attacking polyunsaturated fatty acids (1). In a normal situation, our organism produces AOS continuously (physiological role), but an

Summary

The study of oxidative stress has long been confined to the research laboratory because of the difficulty of assays and the use of sometimes sophisticated techniques that only qualified researchers can use. The recent introduction of sensitive, specific methods that are also quick and suited for routine analysis now enables research and private laboratories to offer the medical world a broad range of tests for evaluating an individual's oxidative stress status. This will make it possible to detect a possible antioxidant deficit and to correct it with an appropriate supplement. It is thus essential to make doctors aware of techniques for measuring oxidative stress in the context of preventing diseases (cancer, atherosclerosis) in which oxidative stress seems to play a major role.

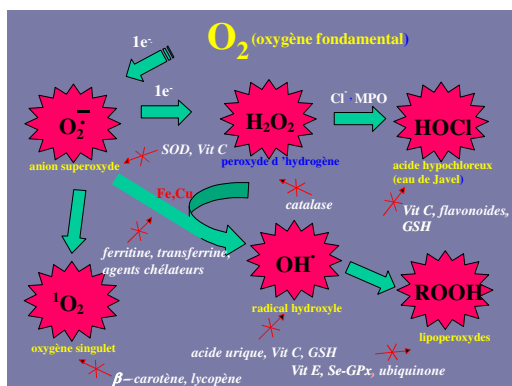
effective antioxidant defence system (consisting of vitamins, enzymes, and oligoelements) regulates this production so as to prevent excessive cell damage (Figure 1).

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Figure 1: Activated oxygen species (AOS) deriving from oxygen and protective systems limiting the toxic effect of these species. GSH: glutathione; CL-; chloride anion; MPO: myeloperoxidase; SOD: superoxide dismutase; Se-GPx: seleno-dependent glutathione peroxidase.



Under some conditions, overproduction of AOS due to activation of various mechanisms can rapidly overwhelm the antioxidant defences: this is called oxidative stress. A growing body of evidence implicates oxidative stress in the cell damage observed in acute inflammation, ageing, cancer, problems linked to ischaemia-reperfusion (organ transplantation), diabetes, and cardiovascular

disease (2). Clinically, the administration of antioxidants is increasingly recommended in order to reduce the toxic effects of AOS. In 15 patients suffering from chronic heart problems, it was found that oral intake of 2 g vitamin C daily for 4 weeks improved significantly the blood flow in the heart (3), thus confirming a preliminary study on 46 patients (4). Two studies, a short-term study focusing on subjects with acute myocardial infarct (61 men, 14 days) and a long-term study of healthy subjects (161 men, 6 years) showed that supplementation with 100 mg (150 IU) vitamin E or 600 mg (900 IU) vitamin E and 600 mg vitamin C can reduce electrocardiographic anomalies significantly as compared to untreated subjects (5,6). In human organ transplantation, several studies have shown that the administration of antioxidants during the period preceding reperfusion of the organ can reduce the plasma concentration of necrosis markers and the intensity of lipid peroxidation and improve the functions of the transplanted organ (7). Several preliminary studies have revealed beneficial effects for N-acetylcysteine (a glutathione precursor) and desferrioxamine (an iron chelator) as agents delaying the progression of HIV infection to AIDS (8). Recently, scientists became aware that antioxidants may also play an important role in preventing the appearance of cardiovascular disease and cancer.

Table 1: Plasma levels of antioxidants and selenium in 13 patients at risk of developing cardiovascular disease (diabetics, hyperlipidemics), selected among the clients of a general practitioner. Results are shown before and after a one-month period during which the subjects took an antioxidant complex (Quatral, Christiaens-Pharma) daily for one month. Each capsule contains: 800 µg vitamin A, 100 mg vitamin C, 30 mg vitamin E, 100 µg selenium, and 15 mg zinc (100% RDI). Plasma levels of selenium and vitamins C and E are significantly increased after antioxidant supplementation.

	Before supplementation	After supplementation	p	Reference values
Vitamin C (µg/ml)	4.19 ± 3.23	8.60 ± 4.05	< 0.002	4 – 9
Vitamin A (µg/ml)	89.49 ± 28.96	83.76 ± 19.23	ns	20 – 80
Vitamin E (µg/ml)	16.85 ± 3.41	20.29 ± 3.75	<0.02	8 – 12
Cholesterol (g/l)	2.28 ± 0.37	2.32 ± 0.31	ns	2 – 2.5
Vit E/cholesterol ratio (mg/g)	7.25 ± 1.28	8.68 ± 1.90	<0.001	4 – 6
Triglycerides (g/l)	1.93 ± 1.03	1.93 ± 1.07	ns	0.3 – 1.7
Selenium (µg/l)	69.03 ± 14.82	91.49 ± 15.37	<0.005	70 – 110
SH proteins (µM)	326.37 ± 54.72	351.85 ± 59.21	ns	340 - 500

Antioxidants and prevention

A healthy and balanced diet (vegetables, fruits, fish, soy oil) should in principle be sufficient to give our organism all the antioxidants and oligoelements it needs to limit maximally the harmful effects of AOS. Yet life in our modern world confronts us with pollution, prolonged exposure to sunlight and various types of radiation, consumption of alcohol or medicines, and smoking. In our organism, situations such as these trigger free-radical chemical reactions to a degree that Nature did not "plan" for. For instance, a puff of cigarette smoke contains approximately 10^{14} AOS. It is well established that blood levels of antioxidants are much lower in smokers than in non-smokers. The situation is becoming more worrisome because we tend to eat less and less healthily and also because changes in cultivated soils are leading to reduced antioxidant intake.

Several major epidemiological studies have shown that low levels of antioxidants or oligoelements (vitamins C and E, selenium) in the blood correlate closely with a higher incidence of cardiovascular disease and cancer. Numerous experimental and epidemiological studies show, furthermore, that the intake of antioxidant vitamins or selenium may reduce the incidence of cancer, cardiovascular disease, and atherosclerosis (9). Several cohort studies tend to confirm these results. In 11,178 men aged 67 to 105 ("*Established Populations for Epidemiologic Studies of the Elderly*", duration: 9 years), Losonczy et al (10) showed that the daily intake of 60 mg vitamin C had no effect on mortality due to cardiovascular disease, but that this risk was significantly reduced in subjects taking both vitamin C and vitamin E. This same observation had been made previously in a study (duration: 20 years) conducted in Great Britain on 730 men and women aged 65 and above (11). Two years ago, the group of Stephens et al (12) presented in *The Lancet* the conclusions of a secondary prevention study (duration: 520 days) called the *Cambridge Heart Antioxidant Study (CHAOS)*", focusing on 2,000 patients aged 55 years or more, **?showing signs of angiopathy? ?scheduled for angioplasty?**, and receiving each day either a placebo or a capsule containing between 266 mg (400 IU) and 533 mg (800 IU) vitamin E. Compared to the

placebo group, the treated subjects showed a significantly lower (75% lower) incidence of nonfatal myocardial infarct. In a randomised double-blind multicentre study, American researchers recently observed 63% fewer prostate cancer cases, 58% fewer colorectal cancer cases and 46% fewer lung cancer cases in subjects taking 200 µg selenium each day (13). An international study called "The European Prevention of Cancer Trial with Selenium", including American, Danish, Finnish, Swedish, Dutch, Belgian, and English researchers, is currently underway to confirm these preliminary results and thus to prove irrefutably that selenium can protect against cancer.

The French SUVIMAX study (*SUpplémentation en Vitamines et Minéraux AntioXydants*) (internet site : <http://www2.cnam.fr/suvimax/presentation>) focusing on 15,000 subjects will provide precious information on the role of antioxidants in prevention. It will evaluate over an 8-year period the possible cardiovascular-disease-preventing effect of a combination of antioxidants taken daily at physiological dosage (30 mg vitamin E, 120 mg vitamin C, 6 mg β-carotene, 100 µg selenium, 20 mg zinc). The final results of this study will not be known until the end of 2002.

Measuring the oxidative stress status: a reality in medical practice

On the basis of all these data, it increasingly appears that by measuring an individual's antioxidant status, a doctor should be able to obtain information that is important to prevention.

Despite an abundant scientific literature, the medical world has remained sceptical regarding the concept and importance of oxidative stress. This is mainly because, until recently, *in vivo* detection of AOS was difficult. AOS have very short half-lives, so they cannot be detected directly (there is one exception, to be mentioned later). Most technologies measure sub-products of the interaction of AOS with their biological substrates, but they have long been used solely, and with many variants, by research laboratories. In recent years, several

companies have developed and marketed standard analytical methods suitable for routine analysis, so that evaluating an individual's oxidative stress status is now an option for doctors. In this article we shall outline some of these methods.

Determination of biological damage

AOS can interact with proteins, DNA, lipoproteins, and polyunsaturated fatty acids to form oxidised derivatives that can be detected in biological samples such as plasma, serum, or urine.

- **Lipid peroxidation**

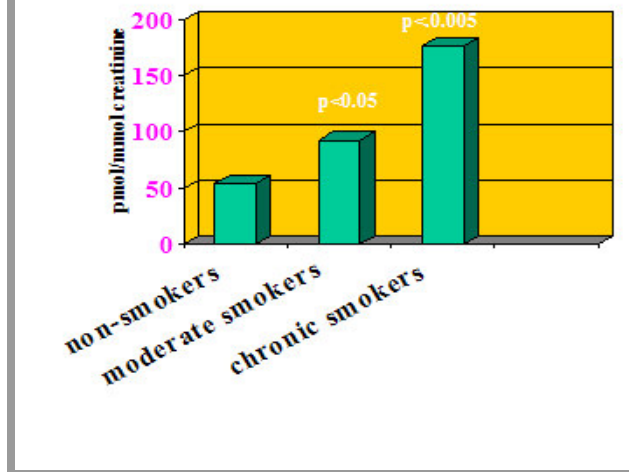
Polyunsaturated fatty acids (RH) such as linoleic acid or arachidonic acid are privileged AOS targets. They are particularly vulnerable to free radicals. First they are converted to lipid peroxides (ROOH), readily detected with a commercial kit (Kamiya Biomedical Co, Thousand Oaks, CA, USA).

Through the action of transition metals (iron, copper), lipid peroxides then decompose into a whole series of sub-products: aldehydes and hydrocarbons. The oldest and most popular method for detecting lipid peroxidation is spectrophotometric detection of malonaldehyde (MDA) by means of the thiobarbituric acid (TBA) test. This method, however, is subject to many artefacts because the presence of haemoglobin in a blood sample or of iron in analytical reagents interferes with the test and leads to results that are totally erroneous and unreliable. Increasingly, the TBA test is being abandoned in favour of high-performance liquid chromatography (HPLC), used to assay MDA in its free form (14). Nevertheless, MDA remains a not-very-representative biomarker of lipid peroxidation, because it constitutes only 1% of all lipid peroxide decomposition products.

More sensitive and reliable is the measurement of another aldehyde, 4-hydroxynonenal (HNE), produced by degradation of ω -6-family fatty acid peroxides. It is routinely measured in the laboratory by HPLC. Clinically, a raised level of HNE has been observed in hepatitis due to ischaemia (15).

In recent years, research has focused on the F2-isoprostanes formed when a hydroxyl radical adds onto a molecule of arachidonic acid. These isoprostanes, the most representative of which is the prostaglandin 8-epi-PGF 2α , are produced in the blood, then excreted in the urine where they are assayed by mass spectrometry. In smokers this test has demonstrated unequivocally the presence of particularly high-level oxidative stress (Figure 2) (16). The recent development of a much simpler and very quick radioimmunoassay will contribute to popularising this measurement, which is still little known (Oxis International, Cutter Circle, USA; importers for Belgium and Luxembourg: BMP, Bruges; Medigal, Villers-Poterie).

Figure 2: Level of 8-epi-PGF 2α , marker of lipid peroxidation, in the urine of smokers and non-smokers. Graph adapted from Reilly et al (16).



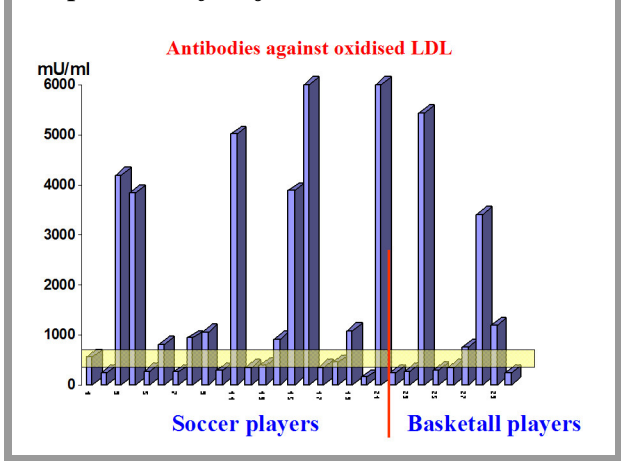
- **Lipoprotein oxidation**

Oxidative damage causes changes in the structure of low-density lipoproteins (LDL), rich in polyunsaturated fatty acids. AOS-induced peroxidation of LDL causes *in situ* formation of aldehydes (MDA and HNE), which can in turn oxidise LDL. These modified LDL are recognised by macrophages, within which they accumulate. This leads to the formation of foam cells which, by accumulating within the interstitial space, contribute to the development of atherosclerosis (17).

In the early 90's, monoclonal antibodies were developed for detecting the presence of MDA and HNE in oxidised lipoproteins (18). With this technique, many researchers evidenced a close correlation between a high level of antibodies against oxidised LDL in the blood and progression of carotid atherosclerosis (19-21). In heart transplant patients, it is well known that deterioration of the coronary arteries may occur, generally in the first year after the operation. One hypothesis proposed to explain this coronary stenosis is accelerated atherosclerosis due to oxidative stress in the endothelium. Just this year the team of Holvoet et al (22) at the University of Leuven used an ELISA to reveal, on a population of 47 heart-transplant patients, a close correlation between high levels of oxidised LDL in the blood and the development of coronary problems during the post-transplant period. A recent study has shown the presence of high levels of these antibodies in diabetic patients (23).

The company Biomedica Gruppe (Vienna, Austria) has recently put on the market an ELISA kit for measuring levels of antibodies against oxidised LDL in plasma or serum (Belgian importer: Alphadia, Wavre). As shown in Figure 3, we have used this test to reveal the presence of oxidative stress in top professional athletes.

Figure 3: Level of antibodies against oxidised LDL in the plasma of Belgian professional athletes (21 soccer players and 9 basketball players). The yellow rectangle represents the extreme normal values (400-600 mU/ml). In 14 players, values far above average indicate the presence of major oxidative stress.



• Protein oxidation

AOS-induced changes in the primary, secondary, and tertiary structures of proteins are responsible for the formation of carbonylated protein derivatives via various mechanisms such as fragmentation and amino acid oxidation. In the presence of dinitrophenylhydrazine (DNPH), these carbonylated derivatives can be detected in biological samples by spectrophotometry, HPLC, or with mono- and polyclonal antibodies. With one of these methods, high levels of oxidised proteins have been observed in the synovial fluid of patients with rheumatoid arthritis (24) and in the plasma of adult patients having developed a respiratory distress syndrome (ARDS) while in intensive care (25). A year ago, a sensitive quantitative ELISA was developed. It is applicable to routine analysis and its results correlate perfectly with those obtained by spectrophotometry. With this method, the mean level of oxidised proteins measured in plasma was 0.75 nmol/mg in 23 intensive care patients versus 0.06 nmol/mg in healthy subjects (26).

• DNA oxidation

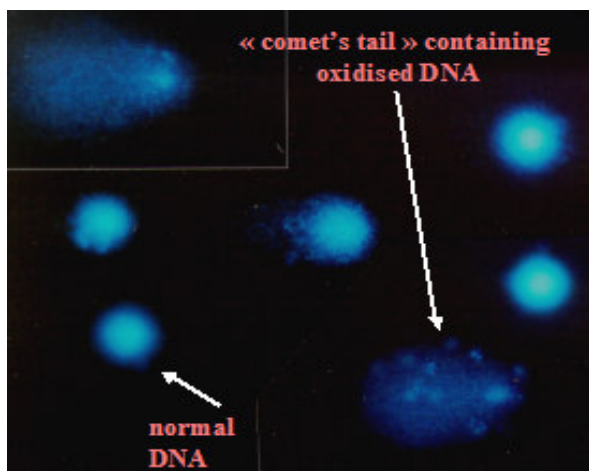
AOS can react with guanine, a constitutive base of DNA, converting it to 8-hydroxy-2' deoxyguanine (8-OH2DG). This altered base is can induce specific mutations leading to cancer development. By means of rather sophisticated HPLC methods it has been shown that the level of these DNA derivatives increases with age in the human brain. These analyses, however, require extreme precautions during sample preparation and are very time consuming. Therefore, they are applicable only in a research laboratory.

To overcome these drawbacks, Collins et al (27) have developed the "COMET" assay for measuring breaks in the DNA of single cells such as lymphocytes. The cells are deposited on an agarose gel, lysed with a detergent, and then treated with a high-salt solution. These operations form nucleotides, so that upon electrophoresis, break-containing DNA will migrate towards an anode as a spot with a "comet's tail" (Figure 4). After staining, the gels are examined by fluorescence microscopy. The relative intensity of the fluorescence measured in the comet's tail is directly

proportional to the frequency of breaks in the DNA. This quick analytical procedure is readily adaptable to routine analysis. Collins et al (27) thus revealed a significantly higher level of lymphocyte DNA oxidation in workers handling carcinogens in a rubber-processing factory than in the administrative staff of the same company. The technique was also used to reveal oxidative stress affecting the DNA of patients with insulin-dependent diabetes.

Recently on the market is an ELISA kit that will make routine DNA analysis even easier. The kit is proposed by the Genox Corporation (Curtland, USA) (Belgian importer: Medigal, Villers-Poterie).

Figure 4: The "COMET" assay for determining oxidised DNA (Photo kindly loaned by Prof A. Collins, Rotwell Research Institute, Aberdeen, UK)



Antioxidants and oligoelements

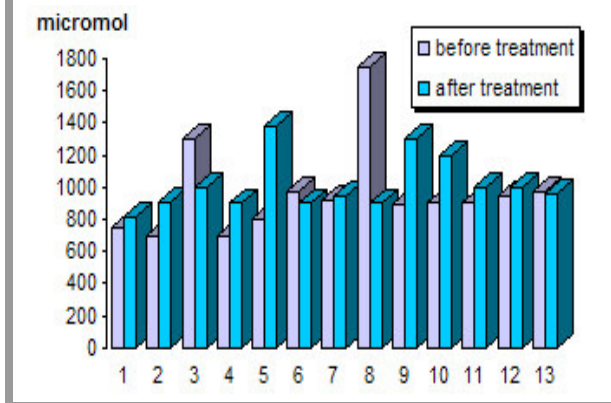
Human plasma is rich in small-sized antioxidants, both hydrophilic (uric acid, ascorbic acid (vitamin C), glutathione, bilirubin) and lipophilic (α -tocopherol (vitamin E), retinol (vitamin A), β -carotene, ubiquinone). Red blood cells, on the other hand, are very rich in antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx). Many animal studies have shown that small-sized antioxidants are usually consumed during oxidative stress. As for the antioxidant enzymes, their levels increase through increased molecular expression when the oxidative stress is minor (adaptation), but

their levels drop when the oxidative stress is too great.

Small-sized antioxidants are easy to assay routinely by spectrophotometry (ascorbic acid, glutathione) and HPLC (ubiquinone, vitamins A and E, β -carotene). For several years there have been commercial kits (Radox Laboratories, Antrim, UK; Belgian importer: Uniprom Diagnostics, Wommel) for assaying SOD and GPx in red blood cells. Just recently the company Oxis International (Cutter Circle, USA) launched a first immunoassay for routine analysis of glutathione peroxidase in plasma. Thanks to these techniques, a great many clinical studies have demonstrated antioxidant consumption during oxidative stress caused by rheumatoid arthritis, AIDS, acute pancreatitis, organ transplantation, septic shock, ARDS, and haemochromatosis (28,29). A decrease in GPx has been observed during the ageing process (30). Many epidemiological studies have also shown that low blood levels of β -carotene and vitamins C and E correlate closely with an increased risk of cancer or cardiovascular disease (31).

Selenium is an essential trace element (oligoelement). It is not itself an antioxidant, but it is viewed as such because it takes part in the constitution and regulation of the detoxifying enzyme GPx, which destroys lipid peroxides. Researchers first became aware of the importance of dietary selenium in 1978. The Keshan region in Eastern China is characterised by a soil very poor in selenium. Over ten million people there contracted "Keshan disease", identified as a fatal congestive cardiomyopathy (32). How did the Chinese authorities eradicate the disease? With dietary supplements of sodium selenite. A year later in New Zealand, a selenium-poor country, researchers were able to correct heart problems appearing during total parenteral nutrition by means of supplementation with selenomethionine (33). Various techniques are used to assay selenium in serum or plasma: X-ray fluorescence, particle-induced X-ray emission spectrometry (PIXE), coupled gas-chromatography-mass spectrometry, and atomic absorption spectrometry with Zeeman correction, this last technique having the advantage that it can be used for routine analysis (Laboratoire Médical du Sud, Namur).

Figure 5: Total antioxidant capacity (TAC) of plasma before and after a one-month period during which 13 patients selected among a general practitioner's clients and at risk of contracting a cardiovascular disease (diabetics, hyperlipidemics) each took one capsule of antioxidant complex (Quatral) daily. The TAC was found to increase in 8 patients out of 13 after antioxidant treatment.



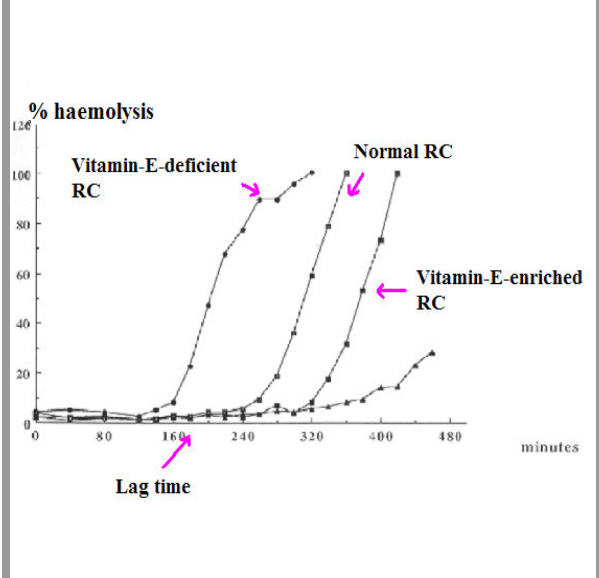
It is thus simple to measure plasma levels of antioxidants and minerals (selenium, but also copper and zinc required to form the active site of SOD). The doctor can thus quickly identify and correct any deficiencies. Table 1 shows how supplementation with antioxidants in quantities near the recommended daily intake (RDI) affects the antioxidant status of plasma.

Total antioxidant capacity

Different antioxidants are affected differently when oxidative stress occurs. It is thus advantageous to have methods for assessing the total antioxidant capacity of a biological sample.

In a test tube, an AOS-generating system is placed in contact with a target (fatty acid, fluorescent probe), and the target's oxidative destruction is monitored as a function of time by spectrophotometry or luminescence (34). If this reaction is carried out in the presence of a plasma sample, the antioxidants present in the plasma interact with the AOS, so that target oxidation does not begin until all the antioxidants are consumed. The latency (lag time before the onset of oxidation) will thus be directly proportional to the amount of

Figure 6: Effect of the intracellular level of vitamin E on the lag time before the onset of haemolysis in red blood cells (RC) exposed to an *in vitro* AOS-generating system.



antioxidants present in the plasma sample. This method is standardised by adding a known quantity of TROLUX, a water-soluble derivative of tocopherol. Studies on patients with lung cancer or a history of acute myocardial infarct have shown that the TAC of their plasma was significantly lower than that of healthy subjects (35,36). Figure 5 shows that antioxidant supplementation at near-RDI dosage causes the TAC to increase (CREDEC, University of Liège).

It is also possible to measure the susceptibility of red blood cells to oxidative stress by exposing them to a system generating AOS at a constant rate and measuring haemolysis as an *in vitro* marker of oxidative stress (Figure 6). The lag time before the onset of haemolysis is directly proportional to the quantity of antioxidants present in the red blood cells. With this technique, Girodon et al. (37) showed that the red blood cells of elderly subjects have significantly lesser antioxidant defences than those of young subjects: the time required for the cells to reach 50% haemolysis was 69 ± 14 minutes in the former population versus 109 ± 12 minutes in the latter.

Iron metabolism

Iron plays a capital role in the initiation and propagation of free-radical reactions, by promoting formation of hydroxyl radicals (OH•). Iron also catalyses the decomposition of peroxidised lipids (ROOH), converting them to alkoxy (RO•) and peroxy (ROO•) radicals that amplify lipid peroxidation. Under physiological conditions, transferrin has a great iron-binding capacity, being only 30% saturated with this transition metal. For this reason, no free iron (in an active state) is found in the blood of healthy subjects. This is why transferrin is considered an important antioxidant. In pathological situations, AOS may cause iron to be released into the blood from its transport proteins (ferritin, lactoferrin), in a form capable of initiating free-radical reactions.

A bleomycin-based assay method can be used to detect this free active iron, as shown in the synovial fluid of patients suffering from rheumatoid arthritis (38) and in the blood of patients undergoing coronary bypass surgery (39). In parallel, the percent iron saturation of transferrin increases to values far above 50%. Easy routine detection of these two parameters thus gives an excellent indication of an individual's oxidative stress level.

Neutrophil activation

Polymorphonuclears (PMNs) or neutrophils play a major role in defending an organism, since they carry out phagocytosis and destruction of foreign microorganisms. The cytotoxicity of these cells is due to their capacity to generate AOS intracellularly, and also to release proteolytic enzymes (elastase, collagenase) and myeloperoxidase (MPO) from their granules.

Independently of this action, PMNs can also be activated by outer stimuli such as protein fragments, cytokines, endotoxins, and complement fragments released in large quantities in various pathological situations (e.g. head trauma). In such cases the products of this activation are released into the extracellular medium, where they attack

healthy tissues and organs and thus trigger acute inflammation.

A high level of MPO and elastase in the plasma is thus an indication of intense leukocyte activation. It provides indirect evidence that AOS have been produced in these pathological situations. This has been demonstrated in patients with ARDS or undergoing coronary bypass surgery (40).

Routine spectrophotometric and immunological assays exist for these two enzymes (elastase: Merck Belgolabo, Overijse, Belgium. MPO: RIA, Pharmacia-Upjohn, Uppsala, Sweden; ELISA, Oxis International, Cutter Circle, USA.)

Electron paramagnetic resonance (EPR)

Electron paramagnetic resonance is the spectroscopic method seen as the best technique for detecting, as directly as possible, free radicals like the superoxide anion, the hydroxyl radical, and lipid radicals. This method measures the energy absorption resulting from the interaction of the radical's free electron with an outer magnetic field produced by powerful magnets. The resulting EPR spectrum can be viewed on a computer screen, analysed, and if needed, quantified, since the height of the signal is directly proportional to the quantity of free radicals in the sample. When oxidised by AOS, ascorbic acid transits through an intermediate stage called the ascorbyl radical. This radical is readily detectable in plasma by EPR. It is thus proposed as a marker of oxidative stress whose level can be measured non-invasively (41), as shown in studies on organ transplant patients and in patients undergoing coronary bypass surgery (42). Yet given their very short half-lives, other oxygenated free radicals cannot be detected without the addition of stabilising molecules (spin traps) to the biological sample (29).

The first-generation EPR instruments were very large, heavy (2 tonnes), and expensive. This precluded their routine use. The recent introduction of smaller, lighter (200 kg), easily movable EPR instruments (Figure 7) has

improved this situation: it is now possible to bring the instrument near the operating room for quick analysis of blood samples taken during surgery (CREDEC, University of Liège). A third generation of even smaller instruments (4 kg) is emerging. We may thus hope that this technology will be increasingly used to determine the oxidative stress of individuals, especially since great progress has been made towards developing spin traps that stabilise free radicals for longer periods.

Figure 7: Photo of the mobile JEOL FR30 EPR instrument for detecting free radicals in blood samples. EPR spectrum of the ascorbyl radical in human plasma.



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