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Antioxidant use in nutraceuticals

Umberto Cornelli, MD*

Stritch School of Medicine, Loyola University, Maywood, IL 60153, USA

Abstract The focus of this contribution is oxidation generated by oxygen and by all other reactive species, with an emphasis on reactive oxygen species. This study considers the different pathways that generate oxidative stress, which is a physiologic process that can become dangerous if becomes excessive and overcomes the reserve of antioxidants. Some of the most important methods to determine oxidative stress in plasma, both in humans and in experimental animals, are discussed; particular attention is given to the d-ROMs test, which detects the hydroperoxides in plasma and is a very simple and reliable method. The antioxidant hierarchy also is discussed to indicate the most powerful physiological antioxidant and those derived from food intake or supplementation. As every antioxidant also can be a pro-oxidant, indications are given about their use and how to avoid the administration of high dosages of a single antioxidant.

Introduction

Antioxidants represent a very large category of products, because many chemical entities may have direct or indirect antioxidant activity. The only official definition of antioxidants is related to "dietary antioxidants."

The definition proposed by the Panel on Dietary Antioxidants and Related Compounds of the Food and Nutrition Board is that "a dietary antioxidant is a substance in food that significantly decreases the adverse effects of reactive oxygen species (ROS), reactive nitrogen species, or both on normal physiological function in human."[1]

ROS and reactive nitrogen species are generated from physiological processes to produce energy and metabolites or to generate defenses against invasive microorganisms. The adverse effect is represented by the oxidative stress (OS) that can arise in case of a lack of antioxidant defense or by an increase of oxidative processes in the body. Oxidative stress has to be a temporary condition, because if it becomes permanent, it may determine a disease. Many different illnesses (such as cardiovascular disease, cancer, and neurological and endocrinological disorders) have been related to OS, which can be either a cause or a consequence of the disease. In any case, no matter what determines its presence, the upregulation of OS is consistent with a pathological condition.

An appropriate equilibrium between oxidation and antioxidants is fundamental to life. Antioxidants and OS can be understood only with the knowledge of the intimate mechanisms that generate the oxidation, and the activity of both endogenous antioxidants and those made available by food intake or supplementation.

Oxygen, free radicals, and reactive species

The presence of O_2 in the atmosphere is a determinant of life, because it makes energy available in the form necessary

The author has no interests in any of the methods used to determine oxidative stress. The author is an executive manager of a company that develops antioxidants for the European Market.

* Piazza Novelli 5, 20123 Milan, Italy. Tel.: +39 02 70121867.
E-mail address: umbertocornelli@cornelliconsulting.it.

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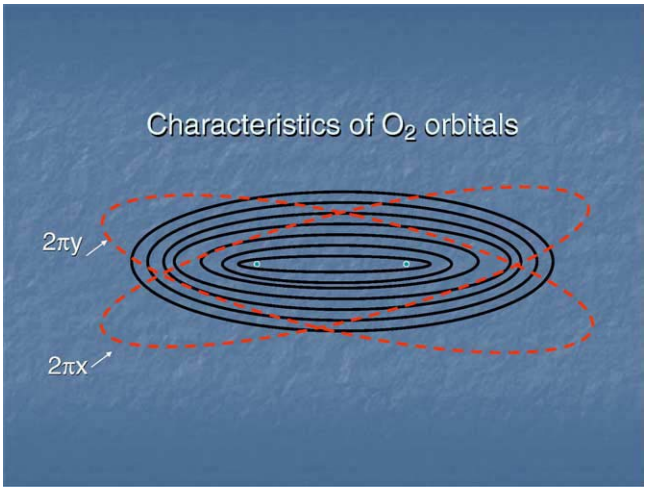

Fig. 1 Oxygen with an unpaired orbital (2πy and 2πx) which determines the bi-radical nature.

for the living such that 1 mol of O_2 may generate 3 mol of adenosine triphosphate (ATP).

Atomic oxygen (O) is formed by a nucleus containing eight protons (the positive charges) and eight, nine, or ten neutrons (with no charge), which constitute the so-called nucleons. Three natural isomers of atomic oxygen exist, which may contain 16, 17, or 18 nucleons and are represented as ^{16}O, ^{17}O, and ^{18}O, respectively.

These three different types of natural isomers are present in the following respective percentages: 99.76%, 0.04%, and 0.2%. Despite these differences in the number of nucleons, the number of electrons (e^-) rotating around the nucleus is always eight. The e^- rotates in five different orbitals represented as 1S, 2S, and 2Pz, which contain a couple of e^- each, and as $2P_x$, and $2P_y$, which contain only one e^-. Since every element that has a single e^- (unpaired) in an orbital is defined as a "free radical," O is a bi-radical by definition.

Things do not change for the molecular oxygen O_2 (Figure 1), because the combination of two atoms does not allow a compensation of the two extreme "combined orbitals", and, consequently, O_2 also remains a bi-radical and should be represented as $O_2^{\bullet\bullet}$. The convention, however, is to simply use the symbol O_2.

As such, O_2 is constantly in search of electrons to compensate for the two unpaired orbital, and this is the essence of "oxidation." Starting as $O_2^{\bullet\bullet}$, the final aim will be to become H_2O, which can be achieved through many different steps, and each step will generate intermediates that are more oxidant than O_2 and are called ROS, as reported in Table 1.

It is known that O_2 is potentially toxic, which was evident when it was used in premature infants and caused retrolental fibroplasia,[2] or in artificial ventilation, which caused pulmonary lesions[3] because of the formation of ROS.

The term oxidation, however, has been expanded to include every process that ends up with a substrate that loses an e^- or a hydrogen atom (H) that contains one e^-, independently from the presence or absence of O_2. Consequently, every substance that loses an e^- or an H is considered as "oxidized" and every substance that receives an e^- or an H is considered "reduced."

The potential damage of O_2 is related to ROS, which are erroneously defined as "free radicals" and represent the tentative of O_2 to compensate for the orbitals that contain only one e^- with the aim of becoming H_2O, which is the real pacemaker of life.

The definition of free radical as a substance that is potentially toxic is incorrect, because most of the elements in the Mendeélev table are free radicals (85/103 elements are free radicals), whereas the capability to oxidize a biological substrate is a much better determinant of toxicity.

The capacity to oxidize biological substrates is a common characteristic of a large group of substances (see Table 1) which are defined as reactive species (RS).

RS are divided into ROS, reactive chlorine species, and reactive nitrogen species. There are many other RS that can be represented as $C^{\bullet}$, $L^{\bullet}$, or $R^{\bullet}$, depending on the nature of the compound: respectively, carbon, lipidic, and generic radical. The entire body of RS in cells, however, tend to be transformed, at least partially and by subsequent reaction, into ROS, which are considered to be the most important RS. The reason for this transformation of RS into ROS is that the final product of the reaction of an ROS will be H_2O, which has an extremely low toxic value.

Table 1 Some of the main RS divided according to the nature of the substance, free radical or nonradical, and grouped by the element that determines oxidation

Free radicals	Formula	Nonradicals	Formula
Reactive oxygen species			
Oxygen	$O_2^{\bullet}$ [a]	Singlet oxygen	Δ o ΣO_2 [b]
Superoxide	$O_2^{\bullet-}$ [a]	Hydrogen peroxide	H_2O_2
Hydroxyl	$OH^{\bullet}$ [a]	Ozone	O_3
Hydroperoxil	$HO_2^{\bullet}$ [a]	Hypochlorus acid	HOCl
Peroxyl	$RO_2^{\bullet}$	Hypobromous acid	HOBr
Alcoxyl	$RO^{\bullet}$	Organic peroxides	ROOH
Carbonate	$CO_3^{\bullet-}$	Peroxynitrite	$ONOO^-$
Carbon dioxide	$CO_2^{\bullet-}$	Peroxynitrous acid	ONOOH
Reactive chlorine species			
Atomic chlorine	$Cl^{\bullet}$	Hypochloric acid	HOCl
		Nitryl chloride	NO_2Cl
		Chloramines	
		Clorine gas	Cl_2
Reactive nitrogen species			
Nitric oxide	$NO^{\bullet}$	Nitrous acid	HNO_2
Nitrogen dioxide	$NO_2^{\bullet}$	Peroxynitrite	$ONOO^-$
		Peroxynitrous acid	ONOOH
		Alchyl peroxynitrite	ROONO
		Nitryl chloride	NO_2Cl

[a] Intermediate step of the transformation (quenching) of O_2 into H_2O.
[b] Generated by sun radiation.

Scheme 1

$O_2^{\bullet\bullet} + e^- \rightarrow O^{\bullet}_2 + e^- + 2H^+ \rightarrow H_2O_2 + e^- \rightarrow OH^{\bullet} + e^- + 2H^+ \rightarrow H_2O$

oxygen — superoxide — hydrogn peroxyde — hydroxy radical — water

Fig. 2 O_2 quenching.

In Table 1, RS are divided into 2 categories, free radicals and nonradicals, which have in common the capability to oxidize biological substrates. Some of the products belong to two different categories as frequently they are regarded in one category or in the other.

The presence of a large amount of RS in the body generate a condition defined as OS.

Oxidative stress

Oxidative stress is caused by an excess of oxidation and/or a lack of antioxidant defense. As it can damage all the constituents of the body (proteins, lipids, DNA, etc), OS has to be a temporary condition, under strict control by the antioxidant defense network which is represented by a variety of enzymatic and nonenzymatic systems.

There are three schematically different pathways to generate OS: energetic, reactive, and metabolic.

The energetic pathway

The energetic pathway is related to the production of ATP and is developed in the mitochondria. The average caloric amount for human body functions is about 2100 kcal/d.

A quantity of 300 mol of ATP is produced (1 ATP = 7 kcal) to fulfill the daily energetic needs, and 100 mol of O_2 is necessary to produce this ATP. At least 1% of O_2 escapes the reaction in the form of one of the ROS and oxidizes closer substrates (leakage). As 100 mol of O_2 is used to generate 300 mol of ATP, at least 3 mol of ROS escapes the cascade from O_2 to H_2O as reported in Scheme 1 (Figure 2).

Four e^-'s are involved in this process, and ROS that is formed in each step can escape the process directed to the formation of water. This event is known as "leakage" and is proportional to the production of ATP.

This cascade of reactions proceeds regularly and rapidly through a series of steps (enzymatic and nonenzymatic) as reported in Scheme 2 (Figure 3).

This cascade indicates that an increase in superoxide dismutase activity results in a concomitant increase in H_2O_2 which can diffuse through biological membranes. As all the reactions of Scheme 2 have to proceed concomitantly, lack of coordination of the system may cause OS by leakage. Exhaustion of catalase and/or peroxydase does not result in the final quenching of $OH^{\bullet}$ into H_2O.

As example, in Down syndrome superoxide dismutase is very high because the gene for its code is in chromosome 21. These patients produce a large amount of H_2O_2 and are easily under OS as none of the quantities of H_2O_2 can be transformed efficiently into H_2O owing to an alteration of the ratio superoxide dismutase/catalase + peroxidase.[4]

In any cell producing energy, in case the quenching system is not efficient, or even in case of excessive production of ATP, it is possible to generate OS by leakage. As this happens within the matrix of the mitochondria, they are the first structure to be damaged and the energy production will be impaired. The cell will not produce the amount of ATP necessary for its normal activity and undergoes premature aging or apoptosis.

The reactive pathway

The reactive pathway is related to the so-called oxidative burst.

In case of stimulation of a reactive cell (leucocytes, macrophages, and so forth) by bacteria, virus, oxidized lipoproteins, or other substances, a large amount of $O^{\bullet}_2$ will be produced through the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which is located in the cellular membrane of the cells. After dismutation, H_2O_2 is immediately available and in the presence of the enzyme myeloperoxydase and chlorine (Cl^-) is transformed into hypochlorite. Furthermore, a part of H_2O_2 may generate $OH^{\bullet}$ in acidic conditions. This is one of the examples of how RS can be transformed into ROS.

In conclusion, the reactive modality ends up with a burst that produces a large amount of different RS which together with proteases aggresses the environment.

Reactive pathway may follow the stimulation of angiotensin II receptors which activate the NADPH oxidase.[5] Hypertension may generate OS via this mechanism. A further reactive mechanism is related to the oxidized low-density lipoprotein or even to the activity of free cholesterol on macrophages.[6]

The metabolic pathway

There are many metabolic reactions that may generate $O^{\bullet}_2$. The most common is the transformation of arachidonic acid into a prostaglandin, or the production of norepinephrine

Scheme 2

$O_2^{\bullet}$

↓ Superoxydodismutase (SOD) "dismutate"* $O_2^{\bullet}$ into O_2 or H_2O_2

H_2O_2

↓ Fenton's reaction in presence of transitions metals of metalloproteins (Fe, Cu, Zn) or reaction of Harber-Waiss in presence of $O_2^{\bullet}$ and metalloproteins

$OH^{\bullet}$

↓ Catalase (mitochondria), peroxyidase (cytoplasma)

H_2O

Fig. 3 Enzymes and reactions involved in O_2 quenching. *Dismutation is a biochemical process where an identical substrate is transformed into two different substances.

from dopamine. In the cascade of production of uric acid from xantine, ROS are generated from hypoxantine to xantine and in the following step from xantine to uric acid; H_2O_2 and $O_2^{\bullet}$ are formed respectively through the same enzyme, the xantine oxidase.

These last reactions are considered the cause of the reperfusion damage.[7-9] Both reactions need O_2 to complete. As during ischemia the availability of O_2 is extremely low, the tendency is to accumulate locally hypoxantine. When suddenly O_2 becomes available a massive OS is developed. Unfortunately, antiproteases (anticoagulant enzymes) are much more sensible to oxidation than proteases[10,11] such as thrombin, and the consequence is the formation of a thrombus. Oxidative stress facilitates the precipitation of acute ischemic episodes and antioxidants may limit the damage/incidence of acute episodes.[12,13] Oxidative stress is also present in practically every woman under treatment with oral contraceptives, as a consistent OS was shown (internal data of the author). The consequence can be the formation of a superficial thrombus which is one of the more frequent side effects of oral contraceptives.

The propagation of oxidative stress

One of the common issues in the production of RS is called "propagation" which may follow any pathway of RS formation. This is particularly effective in case of fatty acids (L) which are located in the membranes of phospholipids (in cells and lipoproteins) and proceeds according to the following steps.

1. The first oxidation (an H is taken out) transforms L in an alkyl radical ($L^{\bullet}$)
2. After an initial tentative rearrangement (diene formation), a further reaction with O_2 generates the formation of a peroxy radical ($LOO^{\bullet}$).
3. At this moment, the propagation reaction starts because $LOO^{\bullet}$ tears out an H from the closest L. The consequence is the formation of a hydroperoxide (LOOH) and an $L^{\bullet}$.
4. LOOH undergoes the Fenton's reaction, which produces either an alkoxy radical ($LO^{\bullet}$) or an $LOO^{\bullet}$, which can both oxidize the closest L and the reaction propagates.

In other words, once an RS reacts with lipids, the propagation starts which can be quenched only by the so-called chain breaker antioxidant (usually liposoluble antioxidants) such as vitamin E. This is one of the reasons for the presence of vitamin E in the cellular membranes.

The mechanism of propagation is very effective as a defense mechanism when it is oriented toward bacteria or virus membranes, but it may be very inappropriate once it is directed against the host membranes (lipoproteins, endothelial cells, internal membranes, etc).

The most important pathway: the equilibrium

All three pathways are important and it is useless to set up a classification in terms of quantity of RS produced endogenously. As oxidation is fundamental to life, it is necessary, however, to maintain an equilibrium between oxidation and antioxidant capacity in every compartment of the body. Usually, OS is a temporary condition and in case it becomes constant it may generate a disease. The real problem is to understand when OS has to be counteracted to avoid the progression or generation of a given disease.

Oxidative stress can seriously damage molecules such as lipids, DNA, proteins, and so forth, after an imbalance between production/presence of RS and antioxidant defense. The latter consists of the pool of nonenzymatic antioxidants and antioxidant enzymes that have to be present and efficient in that part of the body where the oxidation is underway. Some example may clarify the concept.

Procollagen (PC) has to undergo an oxidation to become mature collagen. By oxidation, the lysine residual of PC becomes allysine and forms a bridge between two different chains of PC. In case of OS, more residuals of lysine are oxidized to allysine and too many bridges are formed between PC polymers, and, consequently, the collagen becomes rigid and anelastic. In this case, antioxidants may control the reaction and allow an efficient production of collagen.

It may be that OS acts as a defense mechanism against bacteria or virus, and in this case OS is a protective "reactive" mechanism. In case of blocking of this reaction with antioxidants, a serious clinical problem can arise. Certain types of bacteria or even metastatic cells protect themselves with an efficient antioxidant system.

It is common knowledge that the activation of macrophages through the oxidative burst is a protective mechanism. It potentially damages the subendothelium, however, and in case of inappropriate control of oxidation it can cause atherosclerosis.

This ambivalence has generated criticism against antioxidants because they may interfere with the protection derived from the oxidative processes. Antioxidant intakes have been analyzed during clinical/epidemiological studies that were focused on some them, usually vitamins C and E, β-carotene, and flavonoids. The results were an alternation of positive and negative outcomes.

For antioxidants, however, what prevails is the skepticism of doctors and the belief of consumers who tend to misuse them.

Only a comment can be addressed to this attitude: *antioxidants have to be used when there are conditions of OS that may generate or amplify chronic diseases.*

Oxidative stress has been implicated in many diseases. Diabetes, cancer, cardiovascular, and neurodegenerative diseases are among the most common, but in many other

Table 2 Some of the most common tests under use for the determination of OS and the relative category from C1 to C4

Method	Type of substance that is determined	C	Ref.
DNA	Deoxyribonucleic acid	1	[14]
SPC	Serum protein carbonyls	1	[15]
LHP	Lipids hydroperoxides d-ROMs test	1	[16]
TBARS	Thiobarbituric acid reacting substances	1	[17]
LNO_2	Nitrolinoleate	1	[18]
MDA	Malondialdehyde	1	[19]
4-HNE	4-Hydroxynonenal	1	[20]
IsoPs	$F_2/D_2/E_2$ isoprostanes	1	[21]
F neuroPs	F_3/F_4 isoprostanes	1	[22]
H_2O_2	Hydrogen peroxide	1	[23]
BH	Breath hydrocarbons	1	[24]
ONOO	Peroxynitrite	2	[25]
PTN	Alpha-phenyl-*N*-*tert*-butylnitrone	2	[26]
AHS	Aromatic hydroxylation of salicylate	2	[27]
TRAP	Total peroxyl radical scavenging antioxidant capacity	3	[28]
TOSCA	Total oxyradical scavenging capacity assay	4	[29]
UA	Uric acid	4	[30]
UAM	Uric acid metabolite allantoin	4	[31]
TEAC	Trolox equivalent antioxidant capacity	4	[32]
FRAP	Ferric reducing ability	4	[33]
ORAC	Oxygen radical absorbance capacity	4	[34]
DMPD	*N*,*N*-Dimethyl-*p*-phenylenediamine	4	[35]
DPPH	1,1-Diphenyl-2-picrylhydrazyl	4	[35]
TRX	Thioredoxine and glutaredoxine	4	[36,37]

TRAP, total peroxyl radical trapping; FRAP, ferric-reducing ability test.

diseases a particular emphasis is given to OS. With the increase of pollution, many other environmental sources such as O_3 and $CO_2^{\bullet-}$ are becoming very active partners for OS, and they are practically out of control.

Despite this threat of equilibrium oxidation/antioxidant defenses, OS was never measured in any of the epidemiological studies, and only in a few cases of acute or chronic diseases. In these conditions, it is hard to draw any valid conclusion on the activity of antioxidants on health status.

Nobody would administer an antihypertensive drug to a patient with a normal blood pressure. At the same time, every doctor will use an antihypertensive drug in case of a hypertensive status. It makes no sense to give antihypertensive drugs to everybody and end up with the conclusion that sometimes they are working and sometimes they are toxic. This raises the question of how to determine OS.

Evaluation of oxidative stress

More than 100 different tests are used for the determination of OS. Most are experimental and some are clinically available. To summarize, the following four categories of test are used to determine OS:

1. Determination of substances that have been oxidized by RS. These tests can be used in blood samples (whole blood, serum, plasma) and sometimes in urine also. They are reported in Table 2 as C1.
2. Determination using "spin traps." These are products of different chemical structures capable of capturing RS. They are based on the determination of electron spin resonance which is the paramagnetic signal derived from an unpaired electron. Spin traps have to be administered, and one of the main concerns is their potential toxicity. For this reason, they are used only experimentally. These tests are reported in Table 2 as C2.
3. Determination through substances that once in contact with RS become fluorescent or luminescent. These tests can be used ex vivo in biological samples and are reported in Table 2 as C3.
4. Determination of the antioxidant capability of the blood. These tests are reported in Table 2 as C4.

The prevalent methods are those regarding biomarkers of lipids, DNA, and protein oxidation or the antioxidant capacity of the body.[38,39]

In general, those products that may be considered a mirror of oxidation such as isoprostanes, hydroperoxides, or oxidized DNA are normally produced as a result of a physiological process also. For this reason, they can be found in the blood in relatively limited concentration, which increases under condition of OS.

There are no comparative studies on the different methods in humans. Consequently, it is very difficult to decide which test can be the ideal one or the most reliable one. The same problem arises for the comparison among tests for the determination of the total antioxidant capacity.

Up to now there are no tests that are recognized as standard, and the suggestion is to use one or two tests (d-ROMs, F2-isoprostanes) and learn how to handle the results.

Particular attention was given by the author to the d-ROMs test[30] which is very simple and has been used also to evaluate the antioxidant activity of some products in patients and healthy subjects. The test is based on the determination of hydroperoxides which are derivatives of oxidized lipids and consequently indicate the OS at cellular level. The unit of the d-ROMs test is Carratelli unit (UCARR; mg/dL H_2O_2). The test is used for the epidemiological study of the metabolic syndrome in Italy by the European Society of Biological Nutrition.

The antioxidant network

Assuming that 1 mol of ROS is the daily byproduct of ATP synthesis, and that, hypothetically, the quenching will

Table 3 Redox potential expressed as E'_o (volt) indicates the difference in potential necessary to shift an e) from the left to the right when the concentration of each member of the redox couple is 1 mol at pH 7[40]

Couple	E'_o (V)	e^-	Site of the reaction
Acetate + CO_2/ piruvate	−0.70	2	Glycolysis/ gluconeogenesis
Succinate + CO_2/ α-ketoglutarate	−0.67	2	Krebs cycle
Acetate/acetaldehyde	−0.60	2	Piruvate dehydrogenase [a]
O_2/O_2^-	−0.45	1	Macrophages/neutrophils
$2H^+/H_2$	−0.42	2	(Potential at pH 7)
Acetoacetate/ β-hydroxibutirrate	−0.35	2	Liver chetogenesys
$NAD^+/NADH+H^+$	−0.32	2	Ubiquitarian coenzyme
$NADP^+/NAPH+H^+$	−0.32	2	Ubiquitarian coenzyme
$FMN/FMNH_2$	−0.30	2	Riboflavine phosphate
2GSH/GSSG	−0.23	2	Intracellular antioxidant
$FAD/FADH_2$	−0.22	2	Mitochondrial complex II
Acetaldehyde/ethanol	−0.20	2	Ethanol metabolism
Pyruvate/lactate	−0.19	2	Anaerobic glycolysis
Oxaloacetate/malate	−0.17	2	Krebs cycle
α-Chetoglutarate + NH_4^+/glutamate	−0.14	2	Glutamate synthesis/ catabolism
Fumarate/succinate	0.03	2	Krebs cycle
$CoQ_{10}/CoQ_{10}H_2$	0.04	2	Mitochondrial complex II/III [b]
Dehydroascorbate/ ascorbate	0.08	2	Ubiquitarian antioxidant
1/2 O_2 + H_2O/H_2O_2	0.30	2	Macrophages/ neutrophils
Fe^{3+}/Fe^{2+}1	0.77	1	Fenton's reaction (ubiquitarian)
1/2 O_2 + $2H^+/H_2O$	0.82	2	Mitochondria

e^- represents the number of electrons that are transferred.
NAD(P) indicates nicotinamide adenine diphosphonucleotide (phosphate); FAD, flavine adenine diphosphonucleotide; FMN, flavine mononucleotide; GSSG, oxidized glutathione.
[a] Krebs cycle.
[b] Complexes of the oxidative phosphorylation.

derive from α-tocopherol (vitamin E) only, the total quantity of α-tocopherol needed would be 431 g/d. Such a daily amount of vitamin E is unachievable. This indicates that, to face the problem of oxidation, more than one antioxidant is necessary, and that the complexity of the problem can be solved through an antioxidant network only. Furthermore, antioxidants have to be present in many parts of the body, and because of this they have different structures and tissue affinities.

For these reasons an antioxidant network is needed.

Once an "antioxidant" has made available its electron (e^-) or a hydrogen atom (H) to another substance, it becomes an "oxidant" which is capable of subtracting another substance the entity (e^- or H) that it has just given. In other words, every antioxidant can become a pro-oxidant.

The combined processes of oxidation and reduction form couples of substances called "redox" and may generate a cascade of reactions with other redox couples such that the final biological activity is determined by all the products formed during this cascade.

To understand the process, it is necessary to underline that in biological systems many couples of product take part in the redox processes. As this process is a cascade of reducing and oxidized products, it seems to be a cycle. Fortunately, an end exists which is represented in cells by the reduced glutathione (GSH) as such or by the prosthetic reduced glutathione of the reducing enzymes catalases, peroxidases, and tioredoxines. Enzymes usually do not act as strong oxidants and in case they are not regenerated they stop their activity.

Redox couples

Biochemical studies have made available a list (Table 3) of the most common redox couples calculating the energy that is necessary to subtract an e^- from the reducing form to transform it into the oxidized form. By standardization the energy is expressed as E'_o (volt, at pH 7) and at 1 mol concentration of each member of the couple.

The couple with the higher E'_o value is capable of subtracting the e^- from any couple with a lower value. As example, GSSG can be regenerated to 2 GSH with a redox potential $E'_o = -0.23$ by the NADPH, which will be transformed into the oxidized $NADP^+$ ($E'_o = -0.32$) or by any other couple with $E'_o < -0.23$.

The redox reactions reported in Table 3, however, are standardized to pH 7 and to 1 mol concentration. When pH and concentration change, the sense of the reaction can also change and it is possible that a high concentration of an oxidized product (antioxidant that has given its e^-) becomes pro-oxidant because the tendency to recuperate the lost e^- increases in parallel to its concentration as an oxidized product.

Furthermore, in the biological environment many couples may be localized in the same place where the oxidative process is underway, and, consequently, the final reaction belongs to the relative concentration of the different products. This means that despite the in vitro activity of the different compounds being defined quite precisely, in vivo they may behave very differently.

A wide number of molecules provide an antioxidant effect directly or indirectly. They are heterogeneous from a chemical point of view, and many different approaches were attempted to create a simple classification.

Table 4 reports a classification according to function and structure criteria.[41,42]

Frequently, ω-3 and ω-6 are represented as antioxidants. The problem is that they are polyunsaturated fatty acids (PUFA) which by definition are more sensible to oxidation than saturated fatty acids. The administration of polyunsaturated fatty acids is one of the methods that clinical

Table 4 Some of the compounds that are part of the antioxidant network in humans

Function/ structure	Type of product
Vitamins	Retinol, vitamin E, vitamin C, nicotinamide, riboflavin, niacin
Fats and lipids	ω-3, ω-6, squalene
Amino acids and thiols	Taurine, L-arginine, histidine, glycine, cysteine; glutamine, methionine, *N*-acetyl cysteine, *S*-adenosyl-L-methionine
Peptides	Carnosine, γ-glutamyl cysteinyl glycine (GSH)
Proteins and enzymes	Albumin, thioredoxin, lactoferrin, transferrin, bilirubin, ceruloplasmin, superoxidodismutase, catalase, peroxidase, metallothionein
Plant-derived products	Polyphenols (derivatives of hydroxycinnamic acid, hydroxybenzoic acid, flavonols [a], flavones [a], anthocyanidins [a], flavanols [a], isoflavones [a], flavanones [a], stilbenes, lignans), glucosynolates, carotenoids (α, β, γ, δ-carotene, lycopene, luthein, xeaxantin, canthaxantin), phytic acid, allicin
Minerals	Zinc, iron, copper, selenium, chromium
Metabolites	Uric acid, lipoic acid

[a] Within the class known also as flavonoids.

pharmacologists use to generate OS. In case subjects treated with "fish oils" have the antioxidant system working properly, they can overcome this OS because the antioxidant system is stimulated and generates an adequate compensation. In this case, patients can take advantage of the use of these polyunsaturated fatty acids. *On the contrary, in case the antioxidant system is not working, the outcome will be an increase of the oxidative damage and relative consequences.* The literature is very rich in data concerning all the products listed in Table 4.

Only a combination of products with large clinical trials, however, will be analyzed in more detail.

Antioxidants: clinical definition

The evidence that 24-hour fasting and tranquility have both a strong antioxidant activity may create some complexity in the definition of antioxidants. In many instances, subjects who have diseases (hypertension, infection, inflammation) or under particular conditions such as menopause may also have OS which can be considered as an epiphenomenon of that given condition. Once the disease (or the symptom and/or the condition) is controlled by therapy the OS may disappear. This means that a product can be "indirectly" an antioxidant. This aspect may further complicate the definition of an antioxidant. A temporary definition could be: *an antioxidant is a product that inhibits the oxidation in vitro and reduces the OS in vivo*.

As we have previously shown the determination of OS is made by many different tests. Most of these are experimental and those available for routine clinical use are capable of detecting some endogenous substances (DNA, lipids derivatives, proteins) that have been oxidized or the total antioxidant capacity of body fluids. All these tests represent a derivatization of the OS and measure different types of substrates. The results that come out from each test cannot be comparable with the others and it is possible that products defined as antioxidant in one test do not have a similar activity in another test. This modifies the temporary definition to: *an antioxidant is a product that inhibits the oxidation in vitro and reduces the OS in vivo, no matter in which way OS is measured*.

Typical compounds with these characteristics include some vitamins, such as vitamins C and E, that which have a direct activity like scavengers and also some indirect activities related to different mechanisms[43,44] that may have an impact on the OS.

None of the studies conducted with vitamins or other compounds (such as polyphenols) can give a precise information about any single product, even after supplementation, because foods provide the intake of many of them altogether. The final activity belongs to the combination of a variety of antioxidants. As a consequence, sophisticated statistical analysis had to be applied to the data to isolate the effect of a given compound. Despite this effort, it is very hard to define the activity of a single product.

A certain amount of antioxidant is derived from food intake. Table 5 shows the data of the Division of Health and Nutrition Examination Survey from 1999 to 2000[45] concerning the US population for some of the most common antioxidants.

Systematic data on selenium (Se) and polyphenols are not available. For Se and polyphenols, however, the range of

Table 5 Dietary intakes of selected vitamins by sex and age

Type of vitamin	Men			Women		
	20-39 y	40-59 y	≥60 y	20-39 y	40-59 y	≥60 y
C (mg)	102 ± 4.5	107 ± 6.0	110 ± 7.5	85 ± 5.9	91 ± 5.3	99 ± 3.8
E (mg)	10.4 ± 0.47	10.4 ± 0.44	9.2 ± 0.45	8.2 ± 0.32	9.1 ± 0.41	7.6 ± 0.24
β-Carotene (RE) [a]	377 ± 36.4	537 ± 51.4	559 ± 47.3	522 ± 69.0	554 ± 47.3	507 ± 34.2
A (RE) [a]	878 ± 40.6	1115 ± 80.2	1117 ± 61.5	961 ± 74.4	945 ± 52.8	997 ± 58.5

Values are shown as mean ± SE; sample size from 641 to 1537 subjects.

[a] RE indicates retinol equivalents: 1 RE corresponds to 1 μg of vitamin A and 6 μg of β-carotene.

intake for the US population is approximately between 20 and 200 μg/d, and 50 and 300 mg/d, respectively.

From the data reported in Table 5, the vitamin C difference between sexes is evident; intakes are higher in men, with the only exception being β-carotene in young men. This may depend on the quantity of food intake; more food usually provides more vitamins, and the data concerning RE indicate that certain types of vegetables and fruits do not fit the taste of young men.

The dimension of SE is such that many subjects are not reaching the recommended dietary allowance (RDA), and, consequently, they need to increase their vitamin intake either with food or with supplements. On the other hand, many subjects are taking very high amounts of vitamins with food. In the latter case, a further intake with supplements could generate the condition of a pro-oxidant effect.

Two considerations are extremely important: the first is that the usual antioxidants taken with food are a combination of many different compounds, and the activity derived from them can be either a sum of effect or a synergism (combination effect); the second is related to the amount of each antioxidant (which is usually very low) in the range of the RDA for the most important and known antioxidants in fluid form (concentration effect). These two prerequisites are respected fully when antioxidant intake is determined by food intake.

The antioxidant hierarchy

One of the most comprehensive classifications of antioxidants is reported in Figure 4, where a sort of hierarchy is established according to the potency of in vitro and the abundance of each class. The most active antioxidants are the endogenous antioxidant systems, which can be stimulated according to need. Classic examples of this category are catalases and peroxidases.

Antioxidants that can be defined as "shock adsorbers" are next in potency, because they are available in blood and tissues, but, unlike the enzymatic antioxidants, their production cannot be stimulated after OS. Albumin, transferin, and uric acid belong to this class.

A third category is represented by the essential antioxidant (vitamins, trace metal, amino acids) and substances that are produced as intermediates for more complex molecules (squalene produced during the synthesis of cholesterol) or are part of a more complex macromolecule (coenzyme Q10 as part of cytochromes).

A fourth category (the largest) is made up of natural compounds, such as carotenoids (which are around 600) and flavonoids/polyphenols (which are around 6000).

In general, the top products are the most potent, whereas those in the bottom are the least potent. Among carotenoids and flavonoids/polyphenols, for instance, there are compounds that have very different activity, and the class, per se, is not a determinant of potency. Within flavonoids there are many different compounds with activities that may change at three to four orders of magnitude.

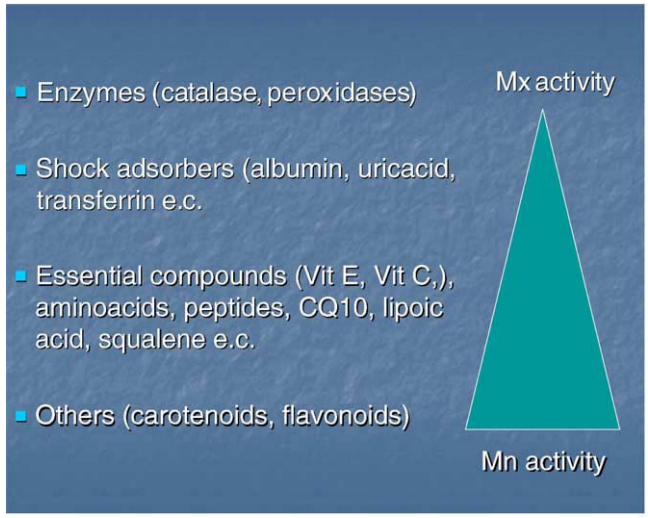

Fig. 4 Antioxidant hierarchy. Modified from Challem J (*Nutr Sci News* 1998;3:352-354). Shock adsorbents represent a circulating antioxidant reservoir.

Another classification is between liposoluble and hydrosoluble antioxidants. The former are located mainly on membranes—either of cells or of lipoproteins—whereas the latter circulate more freely in the blood. Vitamin E, which is highly liposoluble, has a particular affinity for lipoproteins, whereas vitamin C, which is highly hydrosoluble, circulates freely with a minimal protein binding.

The so-called functional classification indicates the preferential localization of antioxidants and is used by the author for the formulation of antioxidant combinations. This classification identifies antioxidants as follows:

- Membrane antioxidants: these are represented by vitamin E, β-carotene, vitamin A, and are known also as lipophilic antioxidants. They have an affinity for membranes of cells and lipoproteins (low-density lipoprotein, very low density lipoprotein, high-density lipoprotein).
- Circulating antioxidants: these consist of vitamin C, amino acids, and polyphenols, which are also known as hydrophilic antioxidants. They are not heavily bound to proteins and may circulate freely in body fluids.
- Cytosol antioxidants: these are produced by cells. Members of this class are lipoic acid, squalene, coenzyme Q10. They are intermediates for the synthesis of endogenous molecules or macromolecules (cytochromes).
- System antioxidants: these are trace metals (such as Se and Zn) or amino acids (such as L-cysteine). They are also components of the antioxidant systems. One of the amino acids of GSH, which is one the most powerful endogenous antioxidant systems, is selenocysteine.

Another classification may consider the direct or indirect activity of the compounds.

Table 6 Some of the most important clinical/epidemiological studies related to antioxidant combinations in food

Study	Duration (y)	*N*	Outcome	Ref
HPFS [a]	3.5	39,910 M	Reduction of coronary hearth disease comparing quintiles of vitamin E	[52]
ARIC [a]	11	13,136 M-F	Reduction of cholesterol; increased high-density lipoprotein cholesterol control of hypertension can lower atherosclerosis progression	[53]
ATBC [b]	6.1	29,133 M	No reduction in the incidence of lung cancer among smokers with supplementation of α-tochopherol or β-carotene	[54]
AHS [b]	6	30,516 M-F	Fruit consumption protects against lung cancer	[55]
ATBC 4 [a]	6.1	4739 M	The highest quintile of fiber intake (median 34.8 g/d) is related to a reduction of major coronary events	[56]
CNBSS [b]	13	56,837 F	No association between dietary carotenoids intake and lung cancer risk	[57]
FMC [a]	14	4697 M-F	Reduction of coronary heart disease comparing tertiles	[58]
GPS [a]	11	1824 M-F	Hostility may be associated with the risk of myocardial infarction	[59]
HPFS [a,b]	3.5	19,687 M	Reduction of coronary heart disease comparing quintiles of vitamin E; no reduction in risk of stroke	[52]
NHS [a]	12	70,089 F	Vitamin E supplementation is associated with a reduced risk of coronary heart disease	[60]
NHS [b]	6	83,234 F	Consumption of fruit and vegetables high in carotenoids and vitamins may reduce postmenopausal breast cancer	[61]
NHScf	12	14,968 F	The use of specific vitamin E supplements but not specific vitamin C supplements may be related to modest cognitive benefits in older women	[62]
NHS II	8	90,655 F	No evidence that higher intakes of vitamin C and E, and folate in early adult life reduce the risk of breast cancer. Vitamin A including carotenoids was associated with a reduced risk of breast cancer among smokers	[63]
VIP [a]	Case Control	16,517 M-F	This study is part of the WHO for monitoring the trends and determinants in cardiovascular disease. Suggestion of reduction of major coronary events	[64]
EPESE	6	11,178 M-F	Simultaneous use of vitamins E and C is associated with a lower risk of total mortality; use of vitamin E reduces the risk of total mortality	[65]
WECS	24	1556 M	Less coronary artery disease owing to vitamin C >113 mg	[66]
IWHS [b]	7	34,486 F	Reduction of risk of death for coronary heart disease; the activity was determined by vitamin E not taken as a supplement; no activity was associated with vitamins A and C	[67]
CVCEE	10	725 M-F	Reduction of cardiovascular disease	[68]
AHS [a]	5	9364 M-F	Frequent consumption of nuts (containing vitamin E) protects against coronary heart disease	[69]
Rotterdam	6	5395 M-F	High intakes of vitamins E and C are associated with a lower risk of Alzheimer disease; activity is more evident in smokers; high intakes of β-carotene may protect against cardiovascular disease	[70,71]
NECSSo	Case control	2577 F	Higher intake of total vegetables and supplementation of vitamin E, B-complex vitamins, and β-carotene protect against ovarian cancer	[72]
FMCHESd	23	4304 M-F	Diabetes type 2 is reduced by the intake of vitamin E in the diet; no association was evident with vitamin C	[73]
SUVIMAX	7.5	13,017 M-F	Antioxidant supplementation reduces the risk of cancer in men; no risk reduction in women. The baseline β-carotene and vitamin C status was lower in men than in women	[74]
SUVIMAX1	7.5	1162 M-F	No activity on carotid atherosclerosis and arterial stiffness	[75]
ARCSd	6	1353 M-F	No relation between diabetic retinopathy and intake of vitamins E and C from food and from food and supplements combined	[76]
CCS	Case control	4750 M-F	Use of vitamin E and C supplements in combination reduces the prevalence of Alzheimer disease	[77]
CARETa	4	14,120	Reduction of lung cancer for the highest vs lowest quintile of fruit consumption	[78]

Table 6 (*continued*)

Study	Duration (y)	*N*	Outcome	Ref
NHNES III	Case control	15,317 M-F	Antioxidant vitamins may prevent hypertension	[79]
NHNESc	Case control	8808 M-F	Participants with the metabolic syndrome had lower circulating concentrations of vitamins C and E, carotenoids (except lycopene), and retinyl esters	[80]
NCSDC [b,c]	6.3	3405 3692 1074 M-F	Dietary or supplemental intake of vitamins A, C, E, folates, and carotenoids is not associated with bladder risk of cancer; inverse association was found between the intake of vitamins, carotenoids, and dietary fibers, and risk of gastric carcinoma; inverse association with lung cancer is found for both vegetables and fruit intake	[81,82]
ASAP	6	520 M-F	Supplementation with combination of vitamin and slow release vitamin C slows down atherosclerotic progression in hypercholesterolemic persons	[83]
MRC/BHF	5	20,536 M-F	Among the high-risk individuals, antioxidant vitamin supplementation did not produce any significant reduction in mortality, vascular disease, or cancer	[84]
AREDS	6.3	4757 M-F	High-dose formulation of vitamin C, vitamin E, and β-carotene had no apparent effect on the risk of development or progression of age-related lens opacity or visual acuity loss	[85]

The HPFS was about vitamin E intake but subjects were also taking carotenoids and vitamin C.
ARIC, Atherosclerosis Risk in Communities; ATBC 4, α-tocopherol β-carotene cancer prevention (subjects receiving vitamin E or β-carotene supplements were excluded); AHS, Adventist Health Study; CNBSS, Canadian National Breast Screening Study; FMC, Finnish Mobile Clinic Examination; same date was reported for the activity of vitamin E (which reduced both in men and in women the risk of coronary mortality); GPS, Glostrup Population Study (Denmark); IWHS, Iowa Women Health Study; VIP, Västerbotten Intervention Program-Sweden part of WHO MONICA project (monitoring trends and determinants in vascular disease); EPESE, Established Population Epidemiological Study of the Elderly; WECS, Western Electric Company Study; CVCEE, Carotenoids, Vitamin C and E in Elderly; NHSsc is a cohort of NHS to study cognitive function (NHS II was related to breast cancer risk. As NHS has been in progress for many years different sets of data are available); NECSSo, Canadian National Enhanced Cancer Surveillance System, partly about ovarian cancer; FMCHESd, Finnish mobile clinic health examination survey for dietary antioxidant intake and risk of diabetes type 2; SUVIMAX, supplementation of vitamins and mineral antioxidant; SUVIMAX1, structure and function of large arteries; ARCSd, Atherosclerosis risk in Communities study in the cohort of cases who had diabetes type 2; CCS, Cashe County Study, Utah; CARETa, β-carotene and retinol efficacy trial, the placebo arm; NHNES III, National Health and Nutrition Examination Survey; NHNES IIIc, National Health and Nutrition Examination Survey for the part related to circulating concentration of vitamins A, C, and E; retinyl esters, carotenoids, and selenium; NCSDC, Nederland Cohort Study Diet and cancer; ASAP, Antioxidant Supplementation in Atherosclerotic Prevention Study; MCR/BHF, Medical Research Council/British Hearth Foundation Heart Protection Study (a randomized placebo-controlled trial); AREDS, Age-Related Eye Disease Study.

[a] Studies that entered the pooled analysis.[86]

[b] Studies that entered the pooled analysis.[87]

[c] Cases are a subcohort with 6.3 years' follow-up based on a total of 120,852 cases (3405 cases for gastric carcinoma, 3692 cases for bladder cancer, and 1074 cases for lung cancer).

Direct activity refers to the capacity of a molecule to become a chain breaker or quencher, whereas indirect activity may interfere with processes that stimulate the production of RS. Steroid, nonsteroidal anti-inflammatory drugs, statins, and some antihypertensive drugs (such as angiotensin-converting enzyme inhibitors) are examples of indirect antioxidants.

The potency of in vitro can be determined for direct antioxidant only. With the development of new reliable systems for the evaluation of OS in vivo, however, indirect antioxidants also will be classified for potency soon. In the following examples only direct antioxidants will be considered.

Antioxidant combinations (food intake)

The link between high fruit and vegetable intake and reduced chronic disease may be related to antioxidant protection. A 24-hour fasting, however, substantially reduces OS, indicating that food of any type generates a balance between oxidants and antioxidants. In other words, caloric intake increases oxidation, whether it is from fruits, vegetables, or fats. A pool of antioxidants taken for a week at very low dosages (very close to RDA or even less) and in fluid form were shown to reduce OS in healthy volunteers.[46]

A higher dosage of antioxidants or an increase in the intake of antioxidants with food did not substantially modify the oxidative markers, despite a significant increase in α-tocopherol, carotenoids, and vitamin C in serum.[47,48]

Long-term administration (between 12 and 36 months) of vitamins C and E alone or in combination at respective daily dosages of 500 and 182 mg (as RRRA acetate) was not able to modify the antioxidant capacity of plasma[49] measured through total peroxyl radical trapping. Lipoprotein resistance to oxidation, however, was improved in the group taking the association of the two vitamins.

Plasma antioxidant capacity after intake of fruits, vegetables, beverages, and some other foods[50] was determined

using the ferric-reducing ability test. The antioxidant capacity significantly correlated to carotenes, and, surprisingly, the single greatest contributor to the total antioxidant intake was coffee, with 68% of the total capacity, whereas tea, wine, fruits, and vegetables were between 2% and 9% only.[51] Results of the studies that considered the intake of antioxidant vitamins in foods only can be related to the combination of many components that are represented also by polyphenols. Some of the studies that analyzed antioxidant vitamins in food are reported in Table 6 with the relevant outcome.

A pooled analysis[86] of nine studies (the Nurses Health Study [NHS] was divided into two studies) reached the following conclusion: "The results suggest a reduced incidence of major events at high supplemental vitamin C intakes. The risk reduction at high vitamin E or carotenoid intakes appear small." A further pool analysis of eight perspective studies[87] concluded that the combination of vitamins A and C intakes from food only was inversely associated with lung cancer risk, and multivitamins or specific supplements were not adding any advantage either. Dosages of vitamin C that were found to start the risk reduction were greater than 140 mg/d for men and greater than 180 mg/d for women, whereas, for vitamin E, the more evident effect is between 9 and 15 mg/d for both sexes.

In the combination of NHS (77,283 women) and Health Professional Follow-Up Study (47,778 men) studies, higher fruit and vegetable intakes were associated with lower risk of lung cancer in women but not in men, although fruits and vegetables provided protection for both men and women who never smoked.[88]

The Medical Research Council/British Hearth Foundation Heart Protection Study controlled the activity of antioxidants in the protection of a large group of patients (10,629) who had coronary artery disease and who were treated daily with vitamin supplements (vitamin E, 600 mg; vitamin C, 250 mg; and β-carotene, 20 mg). Similar high dosages were used in the Age-Related Eye Disease Study (vitamin E, 400 UI; vitamin C, 500 mg; and I-carotene, 15 mg). The results were not positive in either study. In these last two studies (as in any of the studies reported in Table 9), the OS was measured to determine the real need of an antioxidant therapy.

With high dosages of antioxidants, whether derived from diet and/or supplements, the pro-oxidant condition that could further compromise the clinical condition of some patients cannot be excluded.

The nutritional paradigm

In general, the old trials ended up with positive results with the use of supplements, whereas the new more controlled trials showed an opposite outcome. This can be explained partially by the increase of food intake, and consequently of antioxidants. The failure of the more recent trials to show any positive effects also may benefit from a more appropriate methodology in conducting clinical and epidemiological studies.

The daily intake of antioxidants, however, can be one of the keys in the interpretation of the discrepancies between "old and new" trials. In light of this consideration, the most important concept to underline for antioxidants belongs to the "nutritional paradigm." This concept is reported in Figure 5 and is valid for every element, whether it is a macroelement (proteins, fats, carbohydrates, etc) or a microelement (vitamins, minerals, trace minerals) of nutrition.

Disease is generated when the intake of each element is null or insufficient. Increasing an element's quantity up to daily allowance makes a disease disappear. When the element is given in excess, however, it reaches the toxicity limit. In the case of vitamins and some minerals, RDA is well defined, whereas toxicity limit is sometimes less clear and the tendency is to misuse both in megadoses. Furthermore, each dietary allowance and toxicity limit can be different in healthy people and in patients experiencing a given disease. These areas of uncertainty have resulted in the tendency to increase dosages, because the belief "more is good" prevails, particularly in people who are oriented to self-care.

Most of the epidemiological data presented in this contribution shows that high dosages of antioxidants are not active in preventing chronic diseases, and the few positive results were found with moderation of the dosage and when a combination of products was used. A single antioxidant given at high dosages may show some activity, but for reasons not related to the oxidation processes.

One major problem is the scarce use of tests to determine the OS. Even though they are not very precise, they are still the only possible way to determine whether an antioxidant treatment can be effective or not. Nobody would use an antihypertensive drug in a patient with normal blood pressure. The same rule should apply for antioxidant supplements, however they are combined. The activity of

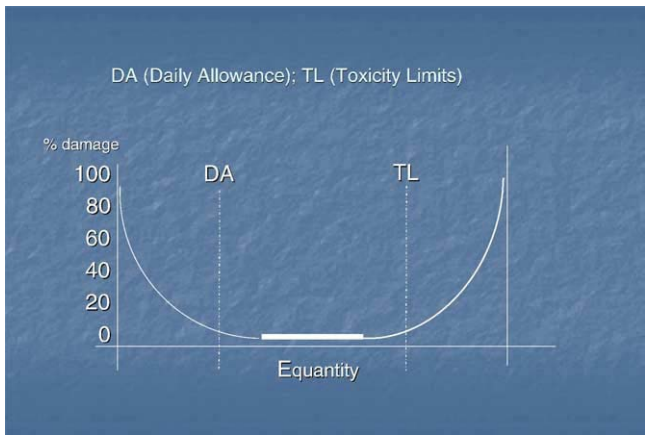

Fig. 5 The nutritional paradigm.

fruits and vegetables for the prevention of cardiovascular disease and cancer indicates that only a combination of vitamins, minerals, and flavonoids taken in relatively low amounts can be considered active.

A few suggestions emerge from the data that have been analyzed in this contribution:

1. To counteract OS, it is not advisable to use only one antioxidant at high dosages, because it is possible that the pro-oxidant activity will prevail over the antioxidant one.
2. It is better to use a combination of antioxidants, and each product should be given in quantities close to the RDA, or if RDA is not determined, at dosages commonly taken with foods.
3. It is necessary to determine OS in blood to avoid administering antioxidants when they are not necessary.
4. For the moment, the suggestion is to use more than one test to measure OS, because each test may address a different compartment of the oxidation. The d-ROMs test can be one test, because it is extremely simple. It is followed by the determination of F2-isoprostanes.
5. The increase in the use of fruits and vegetables or the moderate use of foods rich in antioxidants (such as extra virgin olive oil, tea, wine, coffee, black chocolate, etc) can follow and be a substitute for the supplementation.
6. The new way of eating (as reported in item five) has to be monitored through the control of OS, particularly in patients with chronic diseases.
7. The last suggestion for antioxidants is very simple: "take them if you need them."

Antioxidant combinations (food supplements)

Only a few examples will be reported for this category. Unfortunately, most of the products available on the market are based on fanciful or wild claims.

Every product that is active in vitro may or may not be active in vivo depending upon its availability and the human body environment. The tendency to use large doses of antioxidants can be deleterious, because there are examples in large clinical trials of effects that are the opposite of those expected.[89] The nutritional paradigm shows clearly this type of toxic effect. Consequently, the only way to deal with OS (with any formulation) is to show that a particular combination of antioxidants reduces OS in humans.

Single antioxidant intake is not considered in this contribution, because the usual dosages administered are too high, and, consequently, most of the time they behave as pro-oxidants or because of other pharmacological "nonantioxidant" activities. The author focuses on the combination of antioxidants at low dosages (around RDA when available) and in fluid form. A further consideration (discussed in this contribution) is "compartments of oxidation." This concept indicates that the OS of vessels is not identical to the OS in skin, and different antioxidant products should be used to resolve the specific problem.

Vessels and oxidation

Vessels are present in all tissues and can be considered as the "wall" between blood and tissue. As a consequence, they have to face either the OS generated by "what they are carrying" and the OS generated by " what they are supplying." This means that vessels should be supported by an available and renewable source of different types of antioxidants. The functional classification of antioxidants has been the basis for preparing a combination of natural products to protect the endothelium of vessels from OS.

Endothelium is one of the most sensitive tissues to the OS; it is a producer of RS ($NO^{\bullet}$ in particular) and, consequently, it can be aggressed by what it can produce, by the fluid that it has to transport, and by the tissue that it has to supply.

To provide the most complete antioxidant complex, three antioxidant formulas were tested in a group of volunteers.[46] One formula was composed of membrane antioxidants (vitamin E, vitamin A, and β-carotene) and system antioxidants (Se, Zn, L-cysteine); the second formula was composed of circulating antioxidants (vitamin C, flavonoids from citrus) and cytosol antioxidants (coenzyme Q10 and vitamin B_6); the third formulation was the combination of the two. This last formula is reported in Figure 6.

All the compounds included in the formulas were in very low amount (less than RDA for most compounds). All the formulas were administered once a day in all 14 volunteers in a double-blind crossover design after a 7-day treatment and relative washout. Both dry and liquid formulas were tested and OS was determined with the d-ROMs test (1 UCARR-Carratelli Units- corresponds to 0.08 mg/dL of hydrogen peroxide).

The combination of antioxidants (AR_D Vessel®)

Antioxidant	Dose	RDA
Vit C	30 mg	50
Bioflavonoids (Citrus)	30 mg	-
Vit E	15 mg	150
CQ_{10}	10 mg	-
Vit B_6	1m g	50
Vit A/beta-carotene	480 Re	50
Zn ; Se	5 mg; 48 mcg	35; 50
L-cisteina	10 mg	50

Fig. 6 The most effective combination of antioxidant tested in volunteers in a crossover double-blind study.[46]

Results indicate that only the combination of all antioxidants is really efficient in reducing OS, particularly when products are given as a liquid formulation. These studies demonstrate that supplementation of these types of compounds has to be given *in low concentration and in fluid form…as is the case with foods*.

Oxidation in peripheral arterial disease

Peripheral arterial disease affects approximately 20% of adults older than 55 years and is a predictor of myocardial infarction, stroke, and death due to vascular causes.[90] Typical symptoms, such as leg claudication and walking distance, are the main causes of medical consultation, whereas major coronary and cerebrovascular events are the most frequent outcomes.

Ankle brachial index (ABI) of less than 0.9 measured by Doppler ultrasonography is very useful to identify patients,[91] because it can detect modifications before the appearance of claudication. Other symptoms and laboratory analysis are also helpful for diagnosis, because, hypertension, dyslipidemia, and diabetes frequently are present in peripheral arterial disease. High levels of omocysteine and an increase of d-ROMs test values can be detected in these patients; reduction of blood levels in both can be considered a favorable result of the therapy.

Lifestyle modification (smoking cessation, exercise), vasodilators, and antiplatelet drugs are commonly used, together with antihypertensive, hypolipemic, and hypoglycemic drugs. It is evident that polytherapy must be used with these patients, who sometimes have a big problem with drugs.

Oxidative stress is a common finding in peripheral arterial disease, because of the many concomitant factors that generate damages to endothelial and smooth muscular cells. An antioxidant therapy in oral vials (AR_D Vessel, see Figure 6) was compared to placebo in two groups of subjects (respectively groups one and two) treated for 4 weeks in a double-blind controlled study.

Material and methods

Ankle brachial index, omocysteine, d-ROMs test, and pain-free walking distance (PFWD) with treadmill were taken as a measure for the activity. Thirty-six men (aged between 56 and 68 years) of class Fontaine II were enrolled. Admission criteria were ABI less than 0.9, PFWD less than 150 m, omocysteine greater than 12 μmol/L, and d-ROMS test greater than 380 UCARR.

Ankle brachial index was measured as the ratio between the ankle systolic pressure and the brachial systolic pressure in resting patients. The treadmill was settled at 2 miles/h with 10% inclination; PFWD was used a standard measure. The test was carried out between 8 and 10 AM in an air-conditioned room at 25°C and 30% humidity.

Exclusion criteria were chronic disease (other than peripheral arterial disease) not under adequate therapy. In other words, diabetic, dyslipidemic, and hypertensive patients were admitted to the trial provided that they were under stable therapy. Modifications of the therapy (increase of dosages due to the reduction of the effect) during the trial were considered as a negative outcome.

Laboratory analysis was carried out at baseline and after 4 weeks. After an overnight fasting, blood was taken from the brachial vein in the amount of 5 mL. All the determinations (partly in serum and partly in plasma) were conducted immediately after the collection.

Products, either antioxidants or placebo, were given in a box containing all the therapy for the 4 weeks. A box containing 32 two-phase vials (powder in the cap and fluids in the vial) was distributed to each patient. Each box was labeled with a progressive number from 1 to 56 and contained antioxidants or placebos according to a randomized list. Vials containing ore placebo were identical, and placebo powder consisted of fructose and flavoring. Both products had to be taken once a day in the morning just before breakfast. At the end of the experiment, the patients had to return the box to count the remaining vials for compliance.

Results

- All the patients concluded the 4-week treatment and no relevant side effects were reported.
- Table 7 summarizes the general characteristics of the two groups.
- The two groups were very similar for all the parameters considered.
- After 4 weeks of treatment, the measures chosen for the clinical activity were repeated and the data are summarized in Table 8. The difference between before and after the treatment was statistically significant (t test) for the treatment with an antioxidant only (group one).
- The placebo did not affect favorably any of the items considered. The differences between before and after the treatment were compared and were statistically significant for every variable.

Conclusions

Four weeks of treatment were sufficient to determine an improvement in the vascular condition of the subjects treated with the antioxidant complex. The most interesting data are related to the reduction of OS, because after treatment, most of the subjects showed values less than 300 UCARR, which is a normal value. These results have an impact on the vascular function, as concomitant improvements in ABI

Table 7 General characteristics of the patients treated with the antioxidant complex (group 1) or with placebo (group 2)

Items	Group 1	Group 2	P [a]
Number of cases	18	18	
Age	59 ± 4.5	58 ± 5.1	NS
PFWD (m)	185 ± 80.5	220 ± 100.3	NS
ABI	0.85 ± 0.09	0.85 ± 0.08	NS
Omocysteine (μm/L)	16 ± 3.2	17 ± 4.1	NS
d-ROMs test	410 ± 23.5	400 ± 36.4	NS
Hypertension	14/18	15/18	NS
ACE inhibitors [b]	9/18	10/18	NS
Other therapy	5/18	5/18	NS
Diabetes type II	4/18	5/18	NS
Dislipidemia [c]	12/18	14/18	NS
Statin [b]	7/18	7/18	NS
Other therapies	5/18	7/18	NS

ACE indicates angiotensin-converting enzyme.

[a] Statistical differences were determined according to t test for independent data or χ^2 (Yates correction). NS indicates not significant (or $P > .05$).

[b] A single ACE inhibitor and a single statin were used, the only difference being the dosages, which were never modified during the trial.

[c] Low-density lipoprotein cholesterol >140 mg/dL and/or triacylglycerol >170 mg/dL.

and PFWD had also. These last results were of limited clinical significance, but they indicate that the supply of an antioxidant complex may improve the clinical condition. One expected result of the long-term clinical trial underway is the reduction of the concomitant therapy, which can be determined by a more efficient reactivity of the endothelial cells. To determine the long-term effect on the incidence of major vascular events, more complex trials are necessary. The object of this experiment, however, was to show that OS can be reduced with low-dosage combination of antioxidants. Furthermore, OS seems to be a very sensible marker of the general condition of these types of patients.

Skin and oxidation

The skin represents a very peculiar system in many instances and oxidation is one typical example. Four different compartments can be isolated in the skin: the superficial part (epidermal compartment), which is defined as "brick and mortar"; the connective/elastic fibers of the derma; the so-called gel matrix, which is represented by microvessels and the molecular elements of extracellular matrix (ECM) with the exclusion of connective and elastic fibers; and the cells of the derma (fibroblasts, dendritic cells, mastocytes, etc.).

Table 8 Results after 4 weeks of treatment with antioxidants or placebo

Items	Group 1	Group 2	P [a]
Number of cases	18	18	
PFWD	230 ± 91.4 [b]	200 ± 99.7	<.05
ABI	0.89 ± 0.07 [b]	0.85 ± 0.09	<.05
Omocysteine	12 ± 4.4 [b]	18 ± 4.6	<.05
d-ROMs test	290 ± 18.85 [b]	392 ± 38.4	<.05

[a] t Test for independent data.

[b] t Test for interdependent data.

The brick and mortar

The cells of the granular stratus of the epidermis (epidermocytes) migrate to the surface very schematically, and during the migration they are slowly transformed into corneocytes, which can be defined as mature epidermocytes. Corneocytes are covered entirely by a lipid envelope (Figure 7) which, together with desmosomes, generates the structure called "brick and mortar." The most important function of this compartment is to protect the body from the external environment and to limit transepidermal water loss.

One peculiar feature of the transformation of epidermocytes into cheratinocytes is the loss of receptors for low-density lipoprotein, due to the covering of the lipidic envelope. In such a condition, low-density lipoprotein receptors disappear and cholesterol cannot be taken up by external sources. Consequently, corneocytes have to provide for the synthesis. The synthetic apparatus for cholesterol synthesis is such that about 20% of the total cholesterol of the body is produced by the epidermis.

The necessity to repair this part of the skin after continuous damage, no matter how it can be determined, requires a turnover of new corneocytes, replacing those irreparably

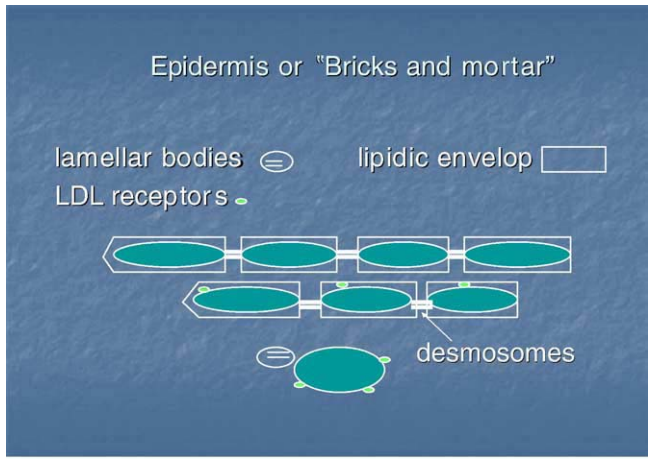

Fig. 7 A schematic representation of the epidermis. The epidermocyte (at the bottom of the figure) produces lamellar bodies that contain lipids (triglyceride cholesterol, cholesterol esters, ceramides, etc). These lamellar bodies are shed out, and by means of enzymes of the ECM, they are used to build up the lipidic envelope. Once the epidermicytes are covered by the lipidic envelope and by cheratin, they become corneocytes (brick). Connection among corneocytes is determined by desmosomes, and lines of cells are very strictly interconnected (mortar).

damaged and lost, and an efficient protection system. Oxidation caused by the external environment is one of the most common and continuous threats. As an example, ultraviolet radiation causes OS, due to the generation of the singlet oxygen, which has a powerful oxidative capacity. One of the most efficient lines of protection of this compartment is represented by squalene.

Squalene is an intermediate for the synthesis of cholesterol and has an antioxidant activity comparable to the common antioxidant vitamins. About 12% to 15% of the sebum is represented by squalene (Figure 8). Squalene (and also vitamin E) is secreted actively by the sebum, and (owing to its liposolubility) diffuses on the epidermis and creates the first natural antioxidant barrier of the skin. After the age of 35 to 40 years, the sebaceous glands drastically reduce the secretion, and the quantity of squalene available for protection becomes insufficient.

Collagen and elastic fibers

Collagen is produced by fibroblasts as procollagen (PC), which is composed of three chains of polypeptides that are shed out from the cells. In the ECM, the combination of two PCs form the mature collagen, which is extremely abundant in the derma (60% of derma). For the conjunction of the two PCs, a fundamental step is necessary (as represented by the oxidation of a residual of lysine to generate allysine), which creates the bridge between the two PCs (Figure 9). In this case, an oxidation is a prerequisite to the formation of collagen.

In case the oxidation is excessive, however, too many bridges are formed between the PC chains, and collagen will become rigid ("old" and anelastic). Aging is characterized by this type of collagen. A similar mechanism can be described for the elastic fibers. They are also produced by fibroblasts as three interconnected polypeptides called proelastin. They are released into the ECM to form elastin (Figure 10). In this case, the formation of bridges also is determined by the oxidation of four lysine residuals to generate desmosine. Parallel to collagen, excessive oxidation of lysine residuals also generates an inefficient collagen that is rigid and "old."

Protection from excessive oxidation can be obtained with β-carotene, which has been shown to be more effective than the other antioxidants tested (coenzyme Q10, vitamin C, and squalene) in the protection against collagen oxidation (Figure 11). The test was conducted in flow cytometry with a low tension of oxygen (130 Torr).

The reason for the better efficiency of β-carotene activity could be because of a more efficient activity than other antioxidants when the oxygen tension is low. This is a condition which is quite common in the derma.

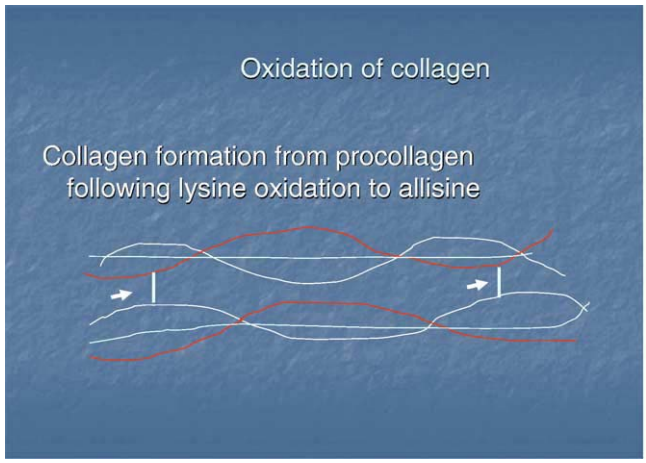

Fig. 9 Schematic representation of the steps of PC oxidation. Three peptidic chains form the PC: 2 PCs are connected by the oxidation of lysine residuals. An excessive oxidation may generate an anelastic and "old" collagen.

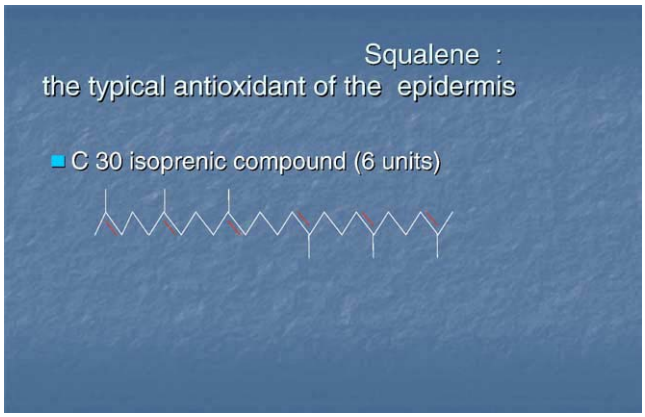

Fig. 8 The structure of squalene. Squalene consists of six isoprenic units. Each unit can donate an electron owing to the instability of the quaternary carbon of the isoprenic unit. Because of its liposolubility, it can diffuse from the area of the sebaceous gland to the proximal epidermis where it behaves as an antioxidant. Consequently, squalene behaves as a chain braker antioxidant.

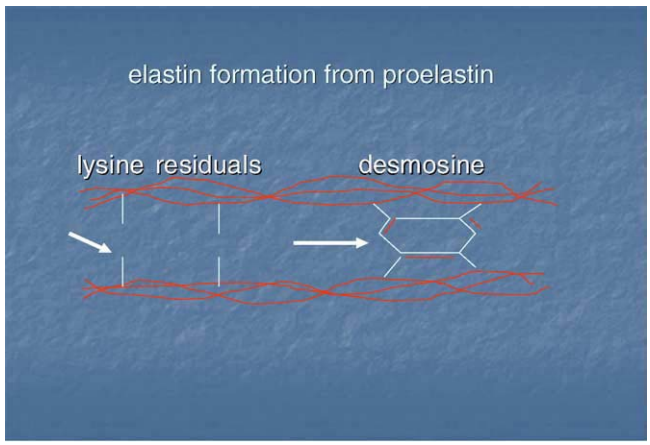

Fig. 10 Schematic representation of the steps of proelastin oxidation. Three peptidic chains form the proelastin polymer. As for collagen, two proelastin polymers are connected by the oxidation of four lysine to desmosine. An excessive oxidation may generate an anelastic and "old" elastin.

Microvessels of the dermis and gel matrix

The importance of vessels in the dermis does not need much explanation, because they represent the input of nutrients and the output of metabolites, damaging substances as it happens for any tissue. Microvessels can be damaged by the tissue that they are supporting and also by the fluid that they are transporting. This complex relation has generated the concept of gel matrix (Figure 12), which indicates the single integrated entity represented by the microvessel (and relative basement membrane), proteoglicans, and water of the ECM.

In other words, gel matrix is the continuity of the endothelial cells in connection to other cells of the dermis (fibroblasts, dendritic cells, melanocytes), collagen, and elastin. Antioxidant defense in this compartment belongs to the antioxidant network, mainly composed of circulating (hydrosoluble) antioxidants.

Cells of the dermis

Each cell of the dermis has a particular function and normally produces ROS as any other type of cell (Figure 13). Dendritic cells, for example, are antigen-presenting cells with tall-like receptors that stimulate the production of ROS, because they behave like macrophages. The mechanism of activation of tall-like receptors is unknown partially, but the consequences are well described in terms of interleukin release and chemotaxis.

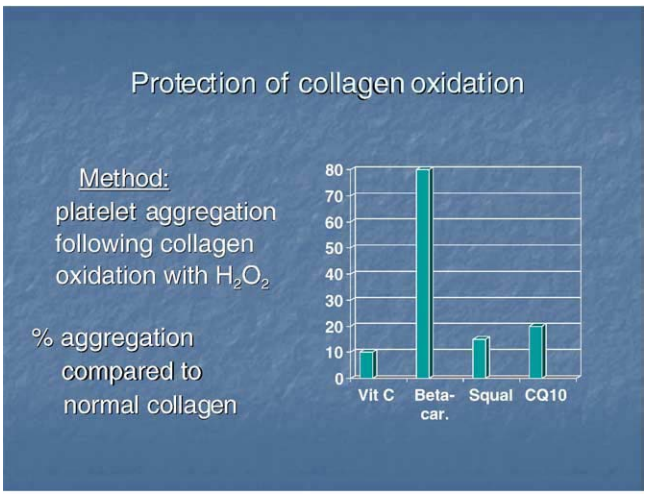

Fig. 11 Percentage of platelet aggregation using oxidized collagen. Protection of collagen with different compounds. Platelet-rich plasma was used for the test. Aggregation was determined by flow cytometry. Data are reported as % aggregation of platelet-rich plasma and intact collagen. To test the activity of compounds, collagen (1 mg/mL) was incubated for 5 minutes with of H_2O_2 (1 μmol). Each antioxidant was added (1 μmol) 5 minutes before H_2O_2 incubation. Higher concentrations (up to 100 μmol) of antioxidants were not more effective for any of the compounds. Beta-car, beta-carotene; Squal, squalene.

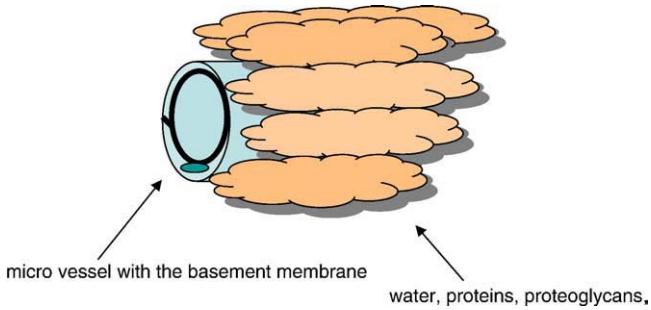

Fig. 12 Schematic representation of gel matrix. Gel matrix is composed by microvessels and by that part of ECM that is in direct contact with the basement membrane. Microvessel permeability has a direct influence on the fluidity of the entire gel matrix as they regulate the fluid exchange and protein permeability. Also, collagen and elastin can be influenced by the gel matrix fluidity.

Fibroblasts also produce ROS as byproducts of ATP synthesis during the synthesis of structural proteins (eg, collagen, elastin, hyaluronic acid). Mastocytes (mast cells) are very reactive cells carrying immunoglobulin E on the membrane and releasing a variety of substances, particularly histamine, heparin, and many interleukins. They are particularly involved in the allergic and inflammatory reaction. These last cells also have a connection with the nervous terminals of the skin and may modulate the receptor activities.

Melanocytes are activated by ultraviolet radiation, and their involvement in OS is not well defined in regards to the quantity that belongs to ATP production.

For all these types of cells, OS follows the paradigm of the balance between intrinsic oxidation and intrinsic antioxidant capacity. The antioxidant support for all these types of cells can be given by cytosol (coenzyme Q10 and squalene), system (Se and Zn), circulating (bioflavonoids), and membrane (β-carotene) antioxidants.

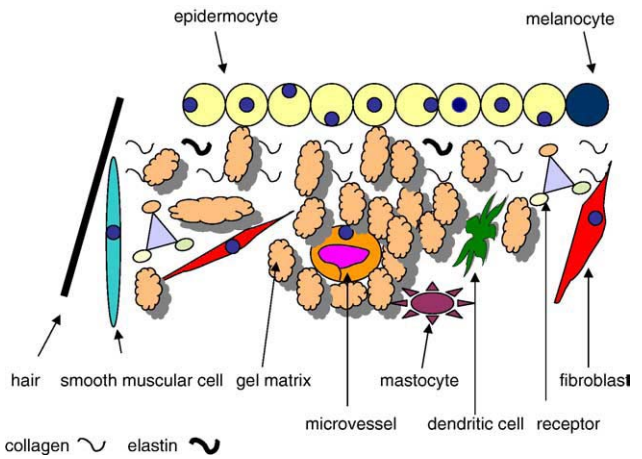

Fig. 13 Schematic representation of the different types of cells of the dermis. The contiguity of all the structures is such that the stimulation of a cell, no matter how it is produced, has an impact on the closest structures generating a cascade that can maintain and amplify the OS.

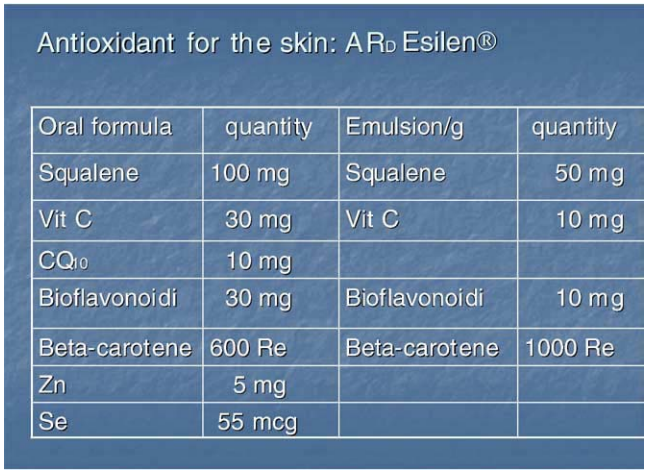

Fig. 14 The different formulas used to increase the elasticity of the skin. Bioflavonoids and β-carotene were derived from *Elaeis guineensis* (red palm oil), whereas squalene was obtained from extra virgin olive oil. Consequently, other minor components of red palm oil and olive oil cannot be excluded and could have some local effect, despite the extremely low concentration.

According to these considerations, two different formulas (Figure 14) have been prepared: one for local application (emulsion) only and another for oral administration (two-phase vials). The emulsion contained squalene (for epidermis protection), β-carotene (for collagen and elastin protection),

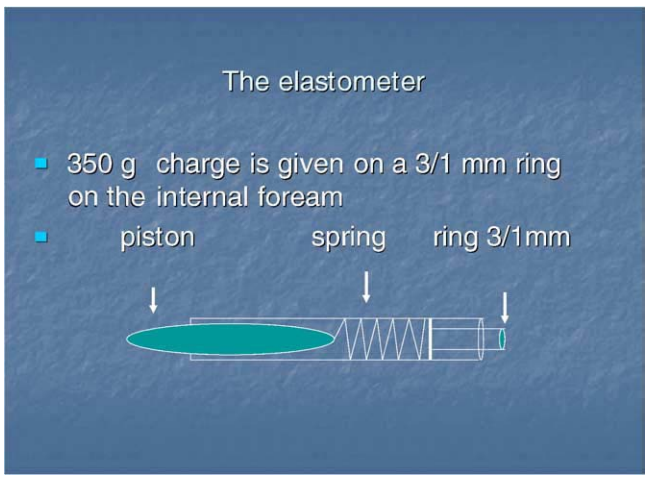

Fig. 15 Schematic representation of the elastometer. The elastometer is a 15-cm device. To perform the test, elastometer has to lean on the medial line of the internal part of the forearm, between the elbow and the wrist. The forearm has to stay on a table and the operator pushes gently the piston. The ring will create a mark on the skin which is 3 mm in diameter and about 1 mm in depth. Time needed for the skin to recover (to become flat again) is a measure of skin elasticity. Soon after the formation of the mark (10-20 seconds), the ring of the skin becomes red (vessel reaction) and, slowly, the color returns to normality. Time (in minutes) needed for the skin to become flat again is between 10 and 25 minutes and is considered a measure of skin elasticity.

Table 9 General characteristics of women treated for the analysis of skin elasticity

Items	Group 1	Group 2
Age	52 ± 6.2	51 ± 7.1
Smoking/total	12/20	11/20
BMI (kg/m^2)	24.7 ± 2.34	24.5 ± 2.17
Housewife/total [a]	10/20	10/20
d-ROMs UCARR [b] (mg/dL H_2O_2)	349 ± 19.7	344 ± 14.7
Elasticity test (min) [b]	22 ± 3.4	21 ± 3.3

[a] Type of activity was considered important, and women were randomized to have in the groups the same number of women working mainly at home or outside as employees.
[b] Each value is the average of three determinations.

and circulating antioxidant (vitamin C and bioflavonoids) for gel matrix defense. The oral formula contained the same product with the addition of systemic (Se and Zn) and cytosol (coenzyme Q10) antioxidants. Dosages for the oral formula were maintained in the order of RDA.

Products were tested in two groups of women in menopause and under OS to determine the activity on skin elasticity. A group of 40 women aged between 48 and 58 years were examined and divided into two groups, with 20 subjects each in a double-blind design comparing placebo and the antioxidant treatment.

The admission criteria were menopause without any other chronic diseases, stability of OS (determined by d-ROMs test >300 UCARR), and stability of skin elasticity (determined with the elastometer) during the run-in period of 1 week (see below). Exclusion criteria were any chronic disease and any chronic therapy. The experimental procedure consisted of a run-in period of 1 week followed by a period of 4 weeks of treatment. During the run-in period, d-ROMs test and elasticity were examined three times.

d-ROMs test was carried out in the serum of overnight fasting women. Blood from the tip of a finger was collected in heparinized minitubes (0.1 mL). Immediately after collection, serum was isolated by centrifugation and 10 μL was used to determine d-ROMs test.[46] In the same day, elasticity was measured in the median part of the

Table 10 Modification of skin elasticity in two groups of women treated for the improvement of skin elasticity

Groups	d-ROMs test UCARR	Right forearm	Left forearm
1 Placebo/placebo	357 ± 23.4	21 ± 6.6	
1 Placebo/AR_D emulsion	357 ± 23.4		18 ± 5.1 [a]
2 AR_D Esilen os/placebo	284 ± 32.7 [b]	17 ± 4.7 [c]	
2 AR_D Esilen os/AR_D Esilen emulsion	284 ± 32.7		14 ± 6.2 [a,b]

[a] $P < .05$ t test: right forearm vs left forearm.
[b] $P < .05$ t test: group 1 vs group 2.
[c] $P < .05$ t test: right forearm group 1 vs group 2.

internal forearm, using the elastometer reported in Figure 15. A compression of 350 g on the skin generated a ring of 3 mm with a depth of 1 mm. The time in minutes necessary for the skin to become normal (flat) is a measure of skin elasticity.

Only women showing the same value of elasticity ±1 minute in the three consecutive determinations (one every other day) were admitted to continue with the trial. Similarly, differences of less than 5% in the first value of d-ROMs were considered as stable values. Forty-five women were analyzed and only five were not admitted because of variation of the d-ROMs test.

Treatment with antioxidants and placebo was given as follows:

1. One group (20 subjects) was treated once a day with placebo orally and with 0.5 mL of a placebo emulsion (seed oil, water, chitosan in the proportion 1:10:1) twice a day. The emulsion was spread on a 5-cm^2 area of the skin in the middle part of the right forearm. The same group was also treated with the emulsion under study (AR_D Esilen emulsion, see Figure 14) spread in the left forearm, following the same modality as for the placebo emulsion.
2. One group was treated once a day orally with an antioxidant formulation (AR_D Esilen see Figure 14) and locally with the placebo emulsion (same modality as before) in the right forearm and with an antioxidant emulsion (AR_D Esilen emulsion) in the left forearm.

With this type of design, it is possible to determine in the same subjects the activity of both the local and oral application of the product, either taken together or separately.

Treatments were distributed according to a randomization list. The formulations used in this trial are reported in Figure 14. Treatments were continued for 4 weeks, and after 4 weeks, all the measures (d-ROMs test and elasticity) were repeated.

Table 9 shows the general characteristics of the women. The groups were very similar for all the items taken into consideration. Table 10, results are summarized for the two groups after the treatment period.

A significant difference (t test, $P < .05$) was found in the two groups. Women treated with placebo only showed both much higher value of d-ROMs test and longer time to recover the compression. The left arm of the women treated orally with placebo, however, showed a better recovery of the compression, indicating that the local treatment with emulsion containing antioxidants may improve the skin elasticity. The d-ROMs test was significantly lower in both groups treated with antioxidants by the oral route. Skin recovery had a better outcome (t test, $P < .05$) in the group treated with the combination of antioxidants by the oral route and locally versus oral antioxidants only.

The complexity of the data indicates that OS reduction proceeded in parallel with an increase of skin elasticity. In conclusion, the antioxidant treatment was effective for local and oral use. The combination of antioxidants applied locally and given orally, however, gives a much more consistent improvement in skin elasticity.

Following the concept of compartment of oxidation, many other different formulations have been prepared and tested in senile dementia, gastrointestinal diseases, and menopause, and it was found that the general concepts are valid and may help in obtaining a more complete understanding of OS.

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