8. ANTIOXIDANTS IN NUTRITION

Victoria Valls-Bellés
Unitat Predepartamental de Medicina (Area: Fisiologia)
Universitat Jaume I
Castelló de la Plana
Spain

8.1 Oxygen and its toxicity

Molecular oxygen is one of nature's most abundant gaseous elements that occurs in the form of diatomic gas (O_2). Despite containing an even number of electrons, molecular oxygen behaves like a paramagnetic molecule, that is, two of its electrons are located in different orbitals and spinning in the same direction with parallel spins. This implies certain connotations of interest in relation to its mechanism of molecular action and more specifically in its oxidative function.

Oxygen has a tendency to complete its electronic structure in its last layer with 8 electrons (octet law). If an oxygen molecule acquires an extra electron in the course of a reaction, it is transformed into another negatively charged paramagnetic and monoradical species known as superoxide anion ($O_2^{\bullet -}$). This is one of the most important free radicals or reactive oxygen species (ROS), responsible for the so-called oxygen toxicity. Its metabolic importance is of particular interest since the discovery of the enzyme superoxide dismutase (SOD), by McCord and Fridovich in 1969, which provided the first *in vivo* evidence of the superoxide anion and the subsequent identification of antioxidant defences. This enzyme is responsible for the dismutation of the superoxide anion to hydrogen peroxide [1].

However, regardless of evolutionary theories and their selection mechanisms, it is evident that oxygen was selected by nature to act as a terminal acceptor of cellular oxidations, thus playing a key role in its reaction with cytochrome oxidase, representing the last link in the electronic transport chain. In this way, cellular metabolism is the result of a series of oxide-reduction reactions whose ultimate purpose is to obtain biological or chemical energy to achieve the performance of different types of biological work such as macromolecule synthesis, active transport, movements muscle, secretory mechanisms, etc.

One of the first known bibliographic reviews on oxygen toxicity is due to Lavoisier who in 1785 already suggested that excess oxygen administration could be as dangerous as its defect. Later,

in the 18th century, other oxygen-derived products, such as H_2O_2 and ozone, were discovered and their effects were shown to be clearly harmful from the beginning of their synthesis [2].

The free radical hypothesis began to be considered as responsible for oxygen toxicity with Michaelis, who published the participation of free radicals as intermediaries of organic oxidations in 1939. Both Michaelis in 1946 and Gilbert and Gerschamn in 1954 provided the first theoretical and scientific basis to explain oxygen toxicity mediated by highly reactive species [3,4,]. Since the 1950s, the participation of reactive species in most of the biochemical processes of biological systems has been accepted. From then on, these species and the proposal of others that would be discovered later, acquire a leading role in the mechanisms of cytotoxic action of oxygen. The implications of this toxicity can be considered in biochemical, toxic-metabolic and pathophysiological processes, whose molecular bases obey or are a consequence of a general mechanism known as oxidative stress. Despite the existence of metabolising and defensive enzymes against oxygen and its free radicals, oxidative stress remains a threatening and continuing danger to living cells [5].

8.2. FORMATION OF REACTIVE OXYGEN SPECIES (ROS) AND OTHER FREE RADICALS IN VIVO.

8.2.1 The concept of free radical

A free radical is defined as any chemical species (atom, molecule or ion) that contains at least one missing electron on an energy level and that is, in turn, capable of existing independently (hence the free term). Free radicals are highly reactive, needing to "steal" or "donate" an electron to another atom or molecule, which is transformed into another free radical, thus generating a chain reaction and producing the so-called oxidative stress.

Free radicals are also known as reactive oxygen species, or ROS, and reactive nitrogen species, or RNS. The combination of these free radicals gives rise to other non-radical reactive species also called reactive oxygen metabolites (ROM) or active oxygen (AO). Among the reactive oxygen species, the ones shown in Table 8.1 are worth mentioning.

As Halliwell indicated in 1996 [6], both ROS and RNS are global terms that, in English, include both radicals and some non-radicals that are oxidising agents of oxygen and nitrogen, and/or are easily converted into radicals, that is, they are reactive species whether or not free radicals. Along with the oxygen and nitrogen radicals there are other derivatives, some centred on atoms of hydrogen, carbon, sulphur, chlorine, etc., which undoubtedly contribute to the propagation and maintenance of

new reactions that lead to the formation of radicals. There are many oxygen species that act as biological oxidants. From the chemical point of view the capacity of each reactive oxygen radical or species is determined by four basic characteristics such as: a) reactivity, b) specificity, c) selectivity and d) diffusivity [7].

Superoxide ($O_2^{\bullet-}$) is the most potent reducing agent and the simple addition of a proton results in the formation of $HO_2^{\bullet}$, thus becoming a very active, selective and specific oxidising agent. $O_2^{\bullet-}$ is not particularly reactive with lipids, carbohydrates or nucleic acids and exhibits limited reactivity with certain proteins. This evidence confirms that $O_2^{\bullet-}$ reacts with proteins that contain metals in its prosthetic group. $^{\bullet}OH$, however, reacts with any molecule that is nearby, without any specificity, so that the danger lies in the functional importance of the cell compartment in which it originates or the molecule to which it attacks. In this way, if it attacks DNA it can produce or generate serious alterations. On the contrary, if the production of the radical takes place in an environment such as plasma and the damaged molecule is an enzyme that is present in large quantities, the actual biological damage will be practically imperceptible.

The three components with the greatest diffusion capacity are $O_2^{\bullet-} < H_2O_2 < {}^{\bullet}OH$, being able to react with molecules that are far from the place of origin and even with the ability to pass through cell membranes (Figure 8.1).

The production of free radicals has to be continuously monitored and maintained at very low concentrations. This is done by the different mechanisms that exist in the organisms forced to live in aerobic environments. For this reason, oxygen species occur in environments sufficiently enclosed to prevent their diffusion or, alternatively, where they can be controlled by the action of defensive enzymes or free radical scavengers synthesised by aerobic cells, that are responsible for their rapid metabolisation to more stable or harmless species [8,9,10].

8.2.2 Sources of free radicals

Free radicals can be both endogenous, generated by the cell itself, or exogenous. They occur naturally as intermediaries or as a product of the numerous oxidative reactions of the cells, as well as through various physical-chemical or biotransformation processes. ROS can also be produced through exposure to environmental oxidants, toxicants, and heavy metals, that can disturb the balance between cell oxidation-reduction reactions, thus altering the normality of biological functions [8,9,10].

8.2.2.1 Endogenous sources

The main endogenous sources of free radicals are:

- Electronic transport chain. In general, it is assumed that mitochondria provide the greatest source of ROS in most cells [11-13]. The main function of the mitochondria is to generate energy, the electronic transport chain being the last link in the combustion of nutrients for obtaining energy in the form of ATP. 95% of the oxygen we breathe is reduced to H_2O by the action of cytochrome oxidase a3, the last link in the electronic transport chain, through a mechanism in which four redox centres participate, providing, in addition, the main source of energy (ATP) to the organism. However, the monovalent reduction of the oxygen molecule results in the formation of three highly reactive species responsible for oxygen toxicity. These species are the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl free radical ($^{\bullet}OH$). Its toxicity is a consequence of its extreme reactivity, which in turn follows from its physical-chemical condition, characteristic of most paramagnetic species. The exception in this case is the hydrogen peroxide (H_2O_2), which, not being a radical, becomes part of important redox reactions to reduce or oxidise to species of greater reactivity (Figure 8.2), [14]. In certain circumstances these ROS can also be produced at the level of complex I and the quinone-semiquinone-ubiquinol complex (Q_{10}) acting as electron acceptors (Figure 8.2) [15]. This situation usually occurs in healthy mitochondria, however, this production can be much higher in certain physiological processes such as ageing. Any physiological situation that involves an increase in mitochondrial respiration will lead to an increase in the formation of free radicals, as occurs during physical exercise. Mitochondria can generate more than 85% of ROS in skeletal muscle tissue [12], but between 1-2% of the O_2 consumed by the organism is naturally converted into $O_2^{\bullet -}$ that will lead to the formation of other ROS after dismutation. It has been estimated that the human being can produce about 2 Kg of superoxide in the body every year and that individuals with chronic inflammatory processes can generate much more [17-21].
- Microsomal electronic transport (hydroxylation reactions). The endoplasmic reticulum, that is, the microsomal fraction of the cell, contains the non-phosphorylating electronic transport system (different from the mitochondrial electronic transport, that is phosphorylating). These systems participate in various hydroxylation and desaturation reactions that produce ROS. A hydroxylation system is constituted by the microsomal enzymes of cytochrome P-450, which are responsible for metabolising xenobiotics. The hepatic microsomal system (Figure 8.3), consists of a flavoprotein called NADPH-cytochrome P450 reductase, and a microsomal

cytochrome, P450. An electronic equivalent is transferred from NADPH to flavoprotein that contains a FAD prosthetic group, which is completely reduced. Subsequently, the electrons are transferred from the reduced flavoprotein to the oxidised form of cytochrome P450 (Fe^{3+}) to give the reduced form P450 (Fe^{2+}), that reacts with (O_2) forming the superoxide ion [22].

- Phagocytic cells. A common fact to all types of inflammation is the infiltration into the affected tissue of cells capable of moving freely. These are mainly leukocytes, neutrophils, monocytes or macrophages. These cells are activated and carry out phagocytosis through an oxygen consumption mechanism, using the NADPH oxidase system directly generating O_2. This consumption can be up to twenty to thirty times higher than that existing prior to activation. On the other hand, phagocytic cells also generate nitric oxide (NO), by the action of nitric oxide synthetase on intracellular arginine, as a defence mechanism. The combination of $O_2^{\bullet-}$ with NO results in the formation of $ONOO^-$, that is capable of inducing lipid peroxidation in lipoproteins, thus destroying cell membranes [23] (Figure 8.4).
- Autoxidation of reduced carbon compounds such as amino acids, proteins, lipids, carbohydrates and nucleic acids also results in the formation of $O_2^{\bullet-}$.
- The catalytic activation of various enzymes of the intermediate metabolism, such as hypoxanthine and xanthine oxidase, aldehyde oxidase, monoamine oxidase, cyclooxygenase, lipoxygenase, nitric oxide synthase, is also a source of free radicals. An example is the deamination of dopamine by the monoamine oxidase that generates H_2O_2 in some neurons and has been implicated with the aetiology of Parkinson's disease [24]. Another example is the enzyme nitric oxide synthase (NOS type I, II, and III) that produces one of the most important radicals in biological regulation in cells: nitric oxide (NO). Another of the most relevant radicals is the superoxide produced by the NAD(P)H oxidase [25]. Xanthine oxidase is another enzyme that participates in the production of ROS, generating superoxide by the oxidation of hypoxanthine to xanthine and then to uric acid [26].

8.2.2.1. Exogenous sources

As previously mentioned, ROS can also be generated by exogenous sources:

- Environmental (electromagnetic radiation, sunlight, ozone and tobacco). Free radicals can be produced in response to electromagnetic radiation, such as gamma rays, which can cleave water and produce hydroxyl radicals [27]. Nitrogen oxides in cigarette smoke cause the oxidation of macromolecules and the reduction of antioxidant levels, which contributes to the

appearance of pathologies in the smoker such as cardiovascular processes and a variety of related cancers, especially lung cancer [27-31].

- Pharmacological (xenobiotics, drugs, etc.). This is the case of anthracyclines, which interact with complex I of the electronic transport chain and induce the formation of free radicals [32,16,33,34].
- Nutritional. ROS are produced in the presence of food contaminants, additives, PUFA, etc. Iron and copper salts promote the formation of free radicals generating H_2O_2. When an individual absorbs a significant amount of dietary iron due to a genetic defect, particularly haeme iron, it becomes a risk factor for cardiovascular disease and certain types of cancer [35]

8.3. Biological damage by ROS

Although ROS have traditionally been observed from a negative point of view for cell function and viability, they can play an important role in the origin of life and biological evolution with beneficial effects on organisms. In recent years, the roles of ROS in signalling and gene expression modulation have been recognised and re-evaluated. In fact, it is not easy to categorise ROS or free radicals as beneficial or harmful molecules. According to Jackson and collaborators 2002, everything depends on the cellular process that is analysed. These authors take two examples, thus, in cell death by necrosis due to ischemia-reperfusion mechanisms, ROS are not beneficial, while in cell death by apoptosis, they can be seen as harmful or beneficial.

The role of ROS in inflammation processes can also be ambivalent. It has been clearly seen as beneficial in the proinflammatory role since it provides an improvement in the immune response following the infection, but in disorders such as rheumatoid arthritis, the inappropriate inflammatory response generated by ROS must be suppressed.

Researchers have concluded that reactive oxygen species play an important role, that can be harmful or beneficial, in changes of modulation of gene expression and cellular function. These changes can be used as biomarkers of oxidative stress.

ROS cause oxidation to biomolecules such as polyunsaturated lipids, cholesterol molecules, carbohydrates, proteins and nucleic acids which are susceptible to being attacked *in vivo* by free radicals (Figures 8.5 and 8.6).

8.3.1 Damage to lipids.

Polyunsaturated fatty acids are highly susceptible to being altered by free radicals, producing lipid peroxidation. Since they have C=C double bonds of the *cis* type, the divinyl-methane structure being repeated within them, each double bond is separated from the successive by an allylic CH_2, which makes them particularly susceptible to attack by free radicals.

The chain of reactions that free radicals produce in fatty acids consists of three essential stages: initiation, propagation and termination (Figure 8.6).

- Initiation reactions. Any species capable of sequestering a hydrogen atom from a carbon chain in a fatty acid (LH) will give rise to a radical located in the corresponding carbon atom ($C^{\bullet}$ or $L^{\bullet}$) of this structure (Figure 8.6). In the fatty acid molecule, a restructuring takes place to form a conjugated diene, a change in the arrangement of the double bonds, together with the carbonyl radical shift (C) to be placed on the next adjacent atom. In lipid peroxidation there are other initiating mechanisms, such as those triggered by the products of this peroxidation, since they are activated species, although they can also be considered as propagating agents in the sense of generating and attacking neighbouring lipid structures. The role of electronic transfer from metal ions is important, since redox reactions between transition metal ions and peroxide compounds play an important role in the formation of free radicals *in vivo*. This is mainly the case of copper and iron, although the reaction of initiation of lipid peroxidation has also been observed with other transition metals. The most important mechanism of action from the point of view of *in vivo* production is the Fenton reaction [36]. Undoubtedly, the species responsible for this initiation in the presence of metal ions is the hydroxyl radical. Both this species and others derived from lipid peroxidation are capable of abstracting a hydrogen atom by themselves [37]. There is another mechanism for initiation of peroxidation reactions, such as the breakdown of chemical bonds by photolytic action [38]. Finally, it is necessary to mention the role of toxic reactions triggered by various xenobiotics such as carbon tetrachloride, or antitumour drugs such as adriamycin. The formation of activated molecular species is involved in the pharmacological mechanism of action of adriamycin, however, the cellular selectivity of this molecule or its effect over normal cells remains to be resolved [32].
- Propagation reactions. Unlike what happens in the processes of initiation and termination, in propagation reactions the number of participating free radicals remains constant. After the extraction of a hydrogen and the molecular readjustment, the radical created has a high reactivity towards O_2 molecules, giving rise to the peroxide radical ($RO^{\bullet}_2$). On the other hand,

the peroxide radical can induce the extraction of another hydrogen from a neighbouring fatty acid molecule, thus initiating another peroxidation cycle. The reaction will end when there is a consumption of substrates or by the conversion of paramagnetic species to radicals or molecules of greater stability and lower reactivity.

- Termination reactions. Termination reactions involve a process in which more than one different type of free radicals participate. There are essentially three termination reactions, which lead to the disappearance of the most reactive species, to gradually replace them with molecules of greater chemical stability. Among these three reactions, two types are distinguished: d) Homotermination and e) and f) cross termination (Figure 8.6). The radicals $2RO^{\bullet}$ and $O^{\bullet}_2$ will combine with each other to form the compound ROOR, that can undergo further oxidation. The choice between one or another reaction path will depend, among other things, on the viscosity, as well as on the concentration of oxygen in the medium. Another factor that determines which reaction will occur is the structure of the substrate. The connotations of termination reactions can be important from the cellular pathophysiological point of view. In no way should it be assumed that the evolution of termination mechanisms ends the distorting danger of free radicals, but quite the opposite. Indeed, in reaction (d) newly-formed links with various biomolecules can be highly destructive for those biological systems in which they are systematically, permanently, and definitively established. As a consequence of lipid peroxidation, there is an alteration in the conductivity, fluidity, permeability and transport of the membranes. If we consider the critical role of polyunsaturated fatty acids as major components of cell membranes, these alterations can be very important [39,40].

8.3.2 Damage to proteins

Proteins and amino acids are also attacked by free radicals. This attack causes changes in cell function, chemical fragmentation and an increase in susceptibility to proteolytic attack. An example is the reversible oxidation of the -SH groups, which is closely linked to oxidative stress in many aspects. There are other types of oxidations of reactive groups of the reversible type, for example, the oxidation of methionine to methionine sulphoxide and its enzymatic reduction back to methionine. However, irreversible oxidation through damage of some amino acid groups is also possible and we find an example in the rupture of the imidazole ring of histidine or tryptophan [41,42].

The possibility of a protein being attacked by a free radical or an oxygen species depends on its composition of amino acids and the accessibility of the oxidising species to them. Certain amino acids strongly react with free radicals, such as methionine and cysteine, both present in some enzymes (lysozyme, pepsin, etc.). This is associated with the loss of biological activity of these enzymes. Proline is another target oxidative stress, especially as a mechanism for breaking peptide bonds. One of the susceptible proteins to this type of destruction is the collagen molecule.

In addition to the oxidation of amino acids, the oxidative stress of proteins is also closely related to the reversible oxidation-reduction of thiol groups, thus, the alteration of the thiol / disulphide status has been shown to have biological consequences such as changes in the kinetic constants and maximum speed of various enzymes.

An example of the importance of protein oxidation is represented by LDL or low density lipoproteins, where histidines and lysines are modified by oxidation, thus causing an alteration in the recognition by receptors [43]. In addition, protein oxidation processes frequently introduce new functional groups such as hydroxyl groups and carbonyl groups, which contribute to altering mobility and protein function. An improvement in the characterisation of these effects has allowed to identify several secondary processes that include fragmentation, cross-linking and splitting, which can accelerate or prevent proteolysis mediated by proteasomes according to the severity of oxidative damage [43].

8.3.3 DNA damage.

The attack of free radicals on DNA generates a series of injuries, which include the breaking of chains and the modification of bases, that produce mutagenesis and carcinogenesis. Oxidative alterations interrupt transcription and replication, increasing the number of mutations. The hydroxyl radical (•OH) attacks DNA, which leads to a large number of changes in purine and pyrimidine bases. Some of these modified bases are considered potentially harmful for genome integrity [44-47,16,48-52].

8.3.4 Cholesterol damage.

The oxidation of cholesterol is of particular biological interest, since cholesterol hydroperoxides and a family of oxidised oxysterols on the β ring of the sterol are produced, as well as derivatives of oxidised cholesterol that are involved in atherosclerosis and cardiovascular disease (Figure 8.7). Currently, there is a lot of evidence indicating that free radicals, lipid peroxidation and oxidative

modification of LDL are involved in the atherosclerosis initiation process. Oxidised LDL is more easily captured by macrophages, which results in the formation of foam cells and induce the proliferation of smooth muscle cells [16] (Chapter 4). It has also been postulated that the presence of oxysterols in the blood may be the result of an effective antioxidant mechanism *in vivo*. This system is based on the possible interaction at the level of blood and various tissues of different oxidising elements with cholesterol, and oxidised derivatives thereof would be excreted via biliary-faecal route [53-58].

8.4 Natural defences

At low concentrations, free radicals are necessary for good cell functioning, being able to act as second messengers stimulating cell proliferation and/or acting as mediators for cell activation. However, an excess of them can accumulate to toxic levels resulting in various actions on the metabolism of the immediate principles, which may be the origin of cellular damage. In order to counteract the toxic action of free radicals, organisms have developed numerous antioxidant defence mechanisms that allow their elimination or transformation into stable molecules [59].

According to H. Sies [10] the biological systems are in a state of approximate equilibrium between prooxidant forces and their antioxidant capacity. The imbalance in favour of the prooxidant action is what is known as oxidative stress. In fact, oxidative damage only occurs when oxidising mechanisms exceed the capacity of defence systems. Therefore, the survival of aerobic cells requires mechanisms that counteract the negative effects of free radicals. According to Halliwell and Gutteridge, antioxidant is any substance that, in the presence or at low concentrations with respect to the oxidisable substrate, significantly delays or inhibits the oxidation of the latter. A good antioxidant is characterised by its high effectiveness, its operational variability and versatility to be able to combine with an important variety of reactive oxygen species. There are antioxidant systems both at physiological and biochemical levels:

- At physiological level: the microvascular system, whose function is to maintain tissue levels of O_2, always within relatively low partial pressures.
- At biochemical level the antioxidant defence can be enzymatic or non-enzymatic; additionally, there are also molecule repair systems.

9.4.1 Antioxidant enzyme system

Aerobic organisms have developed antioxidant enzymes such as: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and DT-diaphorase. SOD is responsible for the $O_2^{\bullet -}$ to H_2O_2 dismutation reaction, will be detoxified by a subsequent reaction catalysed by catalase or GPx, producing H_2O and O_2. Catalase is found mainly in peroxisomes, and its main function is to eliminate the H_2O_2 generated in the beta-oxidation of fatty acids, while GPx will degrade cytoplasmic H_2O_2 [60].

There are other important enzymes that also participate in the defense system, including in the regeneration reactions of Glutathione (GSH), such as GSH reductase, or NADPH-quinone oxidoreductase (DT diaphorase) [61].

- Superoxide dismutase (SOD) (EC.1.15.1.) catalyses the dismutation reaction of the superoxide ion to hydrogen peroxide, which can be subsequently reduced by catalase or glutathione peroxidase (Figure 8.8). Another function of superoxide dismutase is to protect dehydratases (dihydroxide dehydratase acid, aconitase, 6-phosphogluconate dehydratase and fumarate) against inactivation by the superoxide free radical. Four classes of SOD have been identified that contain: copper, zinc, iron, manganese or nickel, as cofactors. In humans there are three forms of SOD: Cytosolic Cu/Zn-SOD, Mn-SOD mitochondrial, and extracellular SOD (EC-SOD) [18]. SOD catalyses the dismutation of $O_2^{\bullet -}$ by successive oxidations and reductions of the transition metal ion at its active site by a ping-pong mechanism with a high reaction rate [62,63]. There is a fourth Ni-SOD enzyme that has been purified from the cytosolic fraction of the mycobacterium *Streptomyces* sp. and *Streptomyces coelticolor*. It is composed of four identical subunits and its amino acid composition is different to those of the three previous SODs [64].
- Catalase (EC 1.11.1.6.) acts similarly to SOD [18]. It reacts at any concentration with H_2O_2 to form molecular oxygen and water, and also shows peroxidase activity with hydrogen donors (methanol, ethanol, formic acid, phenol...), thus protecting the cells from internal peroxides (Figure 8.9). Catalase is a tetrameric enzyme with four identical 60 kD subunits that contain a ferriprotoporphyrin per subunit, its molecular mass being over 240 kD. It is so efficient that can be saturated by H_2O_2 at any concentration [65,64]. It is abundant in mammalian cells and is located in peroxisomes, where it destroys the H_2O_2 generated by oxidases within these organelles [6]. Although catalase is not essential under normal conditions, it plays an important role in the acquisition and tolerance of oxidative stress and cellular response in some cell types [64]. The increased sensitivity of cells enriched with

catalase to adriamycin, bleomycin and paraquat is attributed to the ability of catalase to prevent drug-induced consumption of O_2, either by eliminating H_2O_2, or by direct interaction with the drug [64].

- Glutathione peroxidase (GPx) (EC 1.11.1.8.) is probably the major H_2O_2 scavenger in mammalian cells. In higher organisms, glutathione peroxidase appears largely to supplant the need for catalase. It contains active selenium centres, involved, not only in the elimination of H_2O_2, but also in the metabolism of lipid peroxides (ROOH and H_2O_2). This enzyme uses GSH as a co-substrate to catalyse the reduction of lipid peroxides, which in turn acts as a non-enzymatic antioxidant (Figure 8.10) [18]. At least five isoenzymes of GPx have been found in mammals, their expression levels varying depending on the type of tissue. Cytosolic and mitochondrial glutathione peroxidase reduces lipid peroxides and H_2O_2 at the expense of glutathione (CGPx or GPx1), similarly, glutathione phospholipid hydroperoxidase (GPx4 or PHGPx) is found in most tissues. The latter is located both in the cytosol and in the membrane, and can directly reduce lipid hydroperoxides, fatty acids and cholesterol hydroperoxides that occur in the peroxidation of oxidised membranes and lipoproteins. It is mostly expressed in renal epithelial cells. Cytosolic GPx2 and extracellular GPx3 are poorly detected in most tissues except in the gastrointestinal tract and kidney. Another isoenzyme, GPx5, is specifically expressed in mouse [66]. GPx is considered the main antioxidant enzyme that detoxifies H_2O_2 in animal cells, especially in human erythrocytes, since catalase has a lower affinity for H_2O_2 than GPx. It is also known that cells with decreased GPx are more sensitive to the toxicity of paraquat and adriamycin [67]. GPx and other selenoproteins containing selenocysteine or selenomethionines also functions in the maintenance of the defense against peroxinitrite-mediated oxidations [67]. This enzyme can reduce lipid peroxides, as well as hydrogen peroxide, being a very important enzyme for the maintenance of the structure and function of biological membranes [68].

8.4.1 Non-enzymatic antioxidant system.

A second group of antioxidants are those of non-enzymatic nature, among which various types of molecules of both hydrophilic and lipophilic nature are grouped, whose defensive action will depend in some cases on a direct interaction on the reactive species to yield stable or less reactive complexes. Among them are endogenous antioxidants such as glutathione, uric acid, and certain plasma proteins such as ceruloplasmin and ferritin.

Reduced glutathione (GSH), a tripeptide composed of cysteine, glutamic acid and glycine (γ-Glu-CysH-Gly), is the largest intracellular antioxidant component. Its distribution is universal as it is present in both plants and animals and plays an important role in cellular protection against the toxic effects of free radicals. It occurs mainly in its reduced form in the cells, and a large part of its functions are due to the presence of the reduced thiol group conferred by cysteine. It has an important role as an antioxidant in the cellular defence against free radicals, being able to interact and stabilise hydroxyl, superoxide, and peroxides radicals, in addition to participating in the reduction of other antioxidants such as α-tocopherol and donate hydrogens to repair damaged DNA. On the other hand, it can act as a co-sustrate of antioxidant enzymes such as glutathione peroxidase, as we mentioned earlier. The most important characteristics of GSH are:

- GSH is an exogenous and endogenous antioxidant. The GSH of the diet can be absorbed in the small intestine and can be synthesised again.
- Although the glutathione radical formed by the oxidation of GSH is a prooxidant radical, it can react with another GSH radical ($GS^{\bullet}$) producing GS-SG that is reduced to GSH by the NADPH-dependent glutathione reductase.
- GSH can react with electrophilic components of xenobiotics in a reaction catalysed by glutathione-S-transferase.
- The GSH can be conjugated with NO, thus forming nitrosylated GSH, which will release GSH and NO through a thiol protein system.
- GSH interacts with thiol proteins (glutaredoxin and thioredoxin) that can play an important role in the regulation of homeostasis of the cell's oxidation-reduction system.

GSH is synthesised from glutamate, cysteine and glycine in two stages. In the first stage glutamic acid and cysteine bind to form γ-glutamylcysteine. This reaction is catalyzed by γ-glutamylcysteine synthetase. This step is limited by the availability of cysteine. In the second stage, γ-glutamylcysteine reacts with glycine to form the tripeptide, a reaction catalysed by glutathione synthetase (Figure 8. 11).

Total GSH is regulated by a feedback reaction by γ-glutamylcysteine synthase. The dietary availability of sulphur amino acids may have an influence on the concentrations of cellular GSH [69].

8.5 Antioxidant nutrients

A second group of non-enzymatic antioxidants are those of exogenous origin mainly from the diet such as vitamins (C, E, carotenoids), flavonoids, melanoidins, selenium, etc. [70.71].

8.5.1 Vitamin E

Vitamin E is considered as the main antioxidant sequestering agent of lipophilic radicals *in vivo* in lipid phase and in the external part of lipoproteins. Eight homologues, alpha, beta, gamma, delta-tocopherols and tocotrienols are known and their antioxidant action is given by the group the group -OH of the aromatic ring of the vitamin that can undergo oxidation reactions. One of its most important functions is the inhibition of lipid peroxidation, acting as a "scavenger" of the peroxyl radical ($ROO^{\bullet}$). Another important reaction is the reduction of the alpha-tocopherol radical by other antioxidants such as vitamin C, CoQ and glutathione (GSH). This reaction is very important as it regenerates or saves vitamin E and also reduces the prooxidant character of the vitamin E radical [72,73].

Vitamin E protects cells from peroxidation of membranes and their subsequent degeneration, and prevents oxidative damage of LDL, cellular proteins, and DNA [74]. A diet deficient in vitamin E is related to a reduction in the activity of hepatic catalase, GSH peroxidase, and glutathione reductase, it induces lipid peroxidation in the liver and causes cardiovascular and neurological disorders [75,76].

8.5.2 Vitamin C

Vitamin C or ascorbic acid, is a water-soluble vitamin that is found in a very high concentration in numerous tissues and plasma. It is one of the most potent antioxidants in the aqueous phase, which acts at an extracellular and cytosolic level. Reacts with $O_2^{\bullet -}$, H_2O_2, $ROO^{\bullet}$, $^{\bullet}OH$ and $^1O_2$ by oxidising to dehydroascorbate [77]. It acts synergistically with other scavengers such as vitamin E or urate to regenerate them, reducing them by returning them to their active state. Absorption is a function of intake, greater intake less absorption and vice versa. It can also act as a prooxidant in the presence of transition metals (Cu, Fe), generating the hydroxyl radical. This prooxidant effect of ascorbic acid does not normally take place *in vivo* since in non-pathological situations there is no copper or free iron in extracellular fluids (Figure 8.12) [78,79,80] (see also Chapter 3).

8.5.3 Carotenoids

Carotenoids are divided into two groups, carotenes and xanthophylls. Carotenes such as α or β-carotene or lycopene contain only carbon and hydrogen atoms, while xanthophylls such as

cryptoxanthin, cantaxanthin and lutein have at least one oxygen atom in their structure (Chapter 4). Due to the presence of multiple conjugated bonds, the carotenoids and in particular the 4-oxo derivatives such as astaxanthin and canthaxanthin act as free radical scavengers. They are efficient antioxidants against singlet oxygen and peroxide radical, thus contributing to the body's lipophilic antioxidant defence system. In the presence of peroxyl radicals the oxidative chain ends, as long as the partial O_2 pressures are kept low, otherwise, the oxidative process continues. Therefore, physiological conditions determine the antioxidant or prooxidant character of these compounds (Figure 8.13) [81,82].

The antioxidant capacity of lycopene has long been known as demonstrated by the protection exerted against DNA damage produced by reactive oxygenic species (ROS) [83]. This action, in any case, is also explained by the synergistic effect exerted by the different antioxidants present in foods rich in lycopene such as tomatoes; however, it has been shown that high doses of purified lycopene (30 mg/day) have the same protective effect on DNA [83]. Likewise, this antioxidant role is reinforced by the observation that oxidative stress markers, which usually increase after a meal rich in fat, are attenuated in healthy subjects after ingestion of lycopene [84].

8.5.4. Phenolic compounds

Phenolic compounds, including flavonoids, are a group of natural substances found in the plant kingdom, in fruits, vegetables, seeds, stems and flowers and are, therefore, important constituents of the human diet. The most abundant flavonoids in the diet are flavanols (catechins, proanthocyanidins), anthocyanins and oxidation products derived from them [85, 86].

A mixed western diet provides approximately 1 g of flavonoids per day. Structurally, flavonoids are benzo-γ-pyrones derivatives, their basic structure being diphenylpyranes: two benzene rings linked through a ring-shaped pyrone or heterocyclic pyran [87]. The chemical structure of phenolic compounds is what gives them their ability to act as radical scavengers. The type of compound, the degree of methoxylation and the number of hydroxyl groups are some of the parameters that determine its antioxidant activity. Thus, according to Rice-Evans et al. (1996) [88] the compounds with the highest activity are those with two hydroxyl groups in ortho position in ring B, which gives high stability to the radical that is formed after the free radical capture reaction; those containing a double 2,3 bond in conjugation with the 4-oxo (C = O) in the C ring, and those compounds that have OH- groups in 3 and 5 and the oxo group (C = O) in 4 in rings A and C. They combine with sugars to form glycosides, the most common way in which they are found in nature.

However, it has been seen that the aglycone show a higher antioxidant activity than their corresponding glycosides (Figure 8.14) [89,90]. They can act as hydrogen donors or chelate metal ions such as iron and copper, preventing the oxidation of low density lipoproteins (LDL), which are involved in the pathogenesis of coronary heart disease, inhibit aggregation platelet and protect DNA from oxidative damage. In animal models the effect of epicatechin has been studied in rat hepatocytes and it has been observed that they inhibit lipid peroxidation and increase cell viability. Likewise, catechin prevents the toxic effects of the antineoplastic adriamycin, and the flavonoid fraction of beer, both blonde and black, decreases the oxidation of lipids and proteins, while increasing cell viability, in liver cells after induction of oxidative stress [16].

On the other hand, these compounds present in the diet can favour the defence of endogenous antioxidants through the antioxidant response elements (ARE) found in the promoters of some genes, which are inducible by oxidative stress. Some carcinogenic chemopreventives are thought to act through ARE, by increasing antioxidants and detoxification [91,92].

It has also been proven that flavonoids in combination have greater antioxidant power than in isolation. Recently it has been observed that flavonoids stimulate P-glycoprotein, which participates in the mechanism of cellular defence against the action of xenobiotics [49,55,93,94.95].

8.6 Repair systems

8.6.1 Direct

Reduction of the groups (S-S) of sulfur amino acids in proteins by specific enzymes such as disulfide reductase and sulfoxide reductase.

8.6.2 Indirect

In these repair systems, the molecular damage is first recognised and eliminated or degraded, then the eliminated part is synthesised. This occurs both in the oxidised and lipid peroxide proteins of the carbon chains and in the oxidations of DNA and RNA.

- The oxidised proteins are recognized by proteases and completely degraded to amino acids, proteins are replaced by *de novo* synthesis. The oxidised protein may contain 2 or 3 oxidised amino acids and probably the rest will be reused in the synthesis. If the protein is very oxidised, proteolytic degradation may be inadequate and cross-Linking may occur.

- Lipid peroxidation begins after the extraction of a hydrogen atom in the hydrocarbon chain of polyunsaturated fatty acids, whose vinyl-methane structure represents the target and place of initiation of the peroxidation process. In an aerobic environment interaction of the carbonyl radical ($R^{\bullet}$) with O_2 occurs, leading to the formation of $ROO^{\bullet}$. Subsequently, a new hydrogen (sequential reaction) can be extracted and may give rise to the ROOH that will form the alkoxy radical ($RO^{\bullet}$) by decomposition. This first process is followed by a series of propagation and termination reactions to finally give more stable products, such as malondialdehyde (MDA) and other carbon products that are removed from the cells (Figure 8.5).
- DNA oxidation of DNA. The genetic material is also vulnerable to oxidative damage. Oxidative alterations interrupt transcription, translation and replication by increasing the number of mutations; on the other hand, the increase in oxidative damage is a natural process that under normal physiological conditions produces a modification in the bases, the ratio being of 1/130000 bases in nuclear DNA and 1/8000 in mitochondrial DNA, since the latter is closer to the places where ROS are generated. The DNA repair system is made up of endonucleases and glyosylases [96].

8.7 Oxidative stress and health

There are many pathophysiological processes that are currently associated with the production of free radicals, such as mutagenesis, cell transformation, cancer, diabetes, atherosclerosis, myocardial infarction, ischemia/reperfusion processes, neonate disease (neonatal retinopathy), inflammatory diseases (rheumatoid arthritis, lupus), disorders of the central nervous system (Parkinson's disease, Alzheimer's disease) [97], ageing, etc. In numerous pathologies, reduced levels of antioxidant enzymes or total antioxidants have been observed [98.99].

8.7.1 Oxidative stress and cardiovascular pathology.

Cardiovascular diseases are the leading cause of morbidity and mortality in developed countries. In the year 2,020, according to data from the World Health Organization (WHO), it will be the leading cause of mortality in the world. Cardiovascular pathology is a multifactorial process where obesity, diabetes, hypertension, genetics, dyslipidemia, free radicals, lifestyle, etc. are involved, with hyperlipemia being one of the greatest risks in atrosclerotic processes. atherosclerosis is a very complex process in which LDL and cell proliferation are involved in the endothelium [100,101].

There are different hypotheses to explain the processes associated with the development of atherosclerosis, today one of the most accepted is oxidative modification. LDL is trapped in the subendothelial space where it is susceptible to oxidative modification by resident vascular cells, such as smooth muscle cells, endothelial cells and macrophages. oxidised LDL stimulates monocytic chemotaxis, prevents monocytic outflow and supports the formation of foam cells. Once formed, oxidised LDL also causes endothelial dysfunction and damage, and foam cells become necrotised due to the accumulation of oxidised LDL (Chapter 4, Figure 4.15) [102].

Currently, there is much evidence to indicate that free radicals, lipid peroxidation and oxidative modification of LDL are involved in the process of initiation of atherosclerosis[103,104], giving greater validity to the last hypothesis described [105-107].

8.7.1.1 Oxidation of LDL

In atherosclerotic lesions the existence of different oxidative modifications that take place on lipoprotein has been observed, in addition to the production of reactive oxygen and nitrogen species by vascular cells. Therefore, atherosclerosis has been represented as a state of high oxidative stress characterised by lipid and protein oxidation in the vascular wall. Thus, recent studies have established the presence of oxidised lipids [108], such as peroxides, hydroperoxides, epoxides, etc., in those lesions. Similarly, when reactive species act on cholesterol molecules they produce cholesterol hydroperoxides and oxysterols. In addition, there is evidence of protein oxidation in such lesions [109].

Atherosclerosis and its vascular pathological consequences have been shown to be accentuated with oxidative stress. In the cases in which these oxidation phenomena occur on plasma lipoproteins (LDL), they significantly increase their atherogenic power [110]. These oxidised LDL are responsible for a series of effects such as:

- They are captured more easily by macrophages, which produces their enrichment in cholesterol esters and the formation of foam cells.
- They inhibit macrophage motility in the arterial wall.
- They alters gene expression, inducing cytokine production.
- They can adversely alter coagulation processes, such as the alteration of platelet aggregation.

8.7.2 Oxidative stress and inflammation processes

There has been a growing interest for a long time to establish the exact role that oxidative processes play in the pathogenesis of inflammation, rheumatoid arthritis, asthma, psoriasis and contact dermatitis among other diseases, all of them with a possible common link with oxidative stress [111].

The set of processes associated with the inflammatory response are very complex [112] and often involve the action of ROS. Numerous mediators have been described that could begin to amplify the inflammatory response, such as histamine, serotonin, cytokines and tumour necrosis factor. Inflammation plays an important role in the development of numerous pathologies among which we can mention type II diabetes as well as its associated pathologies such as obesity [113].

In fact, the relationship between inflammation, diabetes and diet has been verified in different studies carried out both on animals and with people. Thus, blood markers of inflammation, such as C-reactive protein (CRP) and interleukin-6, are often considered as predictive parameters of diabetes. A specific case, which illustrates the complexity of these processes, is prostate inflammation, that may contribute to the appearance of cancer in this gland. This risk decreases after ingestion of anti-inflammatory drugs and antioxidant substances [114]. According to some authors, antioxidants may reduce inflammation in the respiratory tract, as well as the increased reactivity that exists in asthmatics [115]. Thus, some studies have shown how antioxidants can reduce the expression of certain types of cytokines (interleukin IL-18) in these asthmatic processes, inhibiting the activity of NF-kappa B, thereby suggesting that reactive oxygen species could regulate interleukin expression [113,115,116]

There is also a growing interest in the role of antioxidants in the control of other diseases with an inflammatory base, such as allergy. It is believed that the antioxidant status of the individual is associated with an increased immune response, although there is no evidence that a lower response to allergens is associated with higher levels of antioxidants [117]. The possible action of antioxidants on immune function has also aroused the interest of researchers on the possible effect of these compounds on pathologies linked to immunological disorders such as multiple sclerosis [118]. Quercetin and other antioxidant polyphenols have a direct relationship with the decrease in inflammation, an effect that would be mediated by the inhibition of proinflammatory cytokines such as tumour necrosis factor [119].

8.8 Mechanisms of ROS elimination

Diet composition plays an important role in oxidative stress as it can contribute to both oxidative damage and antioxidant defence [120]. This partially explains the relationship between diet and some

chronic diseases such as atherosclerosis and cancer. More than 2000 epidemiological studies show that most of the protective effects against a variety of mainly cardiovascular diseases and cancer, correlate with a high intake of fruits and vegetables. Traditionally, nutrition has been recognised as an important factor in the modulation of different diseases and longevity. Diet, particularly through fruit, vegetables, nuts and beverages made from vegetables such as beer [16] and wine, provide antioxidants such as vitamins and other phytochemicals, which are an important exogenous source capable of increasing the cellular response to oxidative stress [121, 122]. In epidemiological studies by Gey et al. (WHO/Proyecto Mónica, 1991) [123], where they determined plasma antioxidants (alpha-tocopherol, ascorbate, vitamin A, carotenoids and selenium) in 16 European populations, the incidence of mortality from ischemic heart disease shows an inverse relationship with plasma levels of alpha-tocopherol ($P = 0.002$). Studies on the intake of fruits and vegetables in Europe show the high difference in consumption between Northern and Southern Europe. These studies have shown a lower incidence of cardiovascular diseases and cancer in Southern European countries, where a Mediterranean diet is consumed, with respect to Nordic countries.

The Mediterranean diet (Chapter 15) is mainly constituted by a high consumption of fruits, vegetables, legumes, unrefined cereals [124,125], low levels of meat and dairy products, and a moderate consumption of wine, beer and fish. Olive oil is a major contributor to this diet as a source of fat. The Mediterranean diet is considered a diet with high antioxidant activity [126-129] and nowadays it is known that fruits, vegetables, olive oil, etc. contain other compounds, even with greater antioxidant activity than vitamins and flavonoids [130,131].

The Rotterdam study investigated the relationship between dietary intake of flavonoids and antioxidant vitamins and the risk of ischemic cerebrovascular accident (CVA) for an average of 6 years, observing that a high dietary intake of antioxidants is associated with a lower risk of CVA. It has been epidemiologically proven that a high dietary intake of fruits and vegetables produces a 50% reduction in the risk of digestive and respiratory tract cancers [12]. These studies support the hypothesis that natural antioxidants from food can protect cells from oxidative stress [132,133].

In addition, we have to keep in mind that not all effects are due to antioxidant vitamins and flavonoids, but other compounds found in foods can indirectly contribute to the reduction of these pathologies. For example, high plasma levels of homocysteine are a risk factor for cardiovascular diseases, while folates reduce homocysteine levels in plasma, therefore, folate from the diet indirectly contributes to reducing the risk of cardiovascular diseases [132].

To reduce or eliminate free radicals, you must follow a diet rich in fruits and vegetables, and include a minimum of four different colours every day, in order to obtain a wide variety of compounds with antioxidant activity [133].

Dietary antioxidants, which include catechins, flavonoids, anthocyanins, stilbenes and carotenoids, demonstrate benefits in the prevention and/or support of therapy in chronic diseases. Antioxidants reshape DNA methylation patterns through multiple mechanisms, including regulation of epigenetic enzymes and chromatin remodelling complexes. These effects may further contribute to the antioxidant properties of the compounds [133-136].

At present, and due to their stability, the most effective antioxidants are polyphenols with different chemical structures, which give them different antioxidant activity [137,138]. They present antioxidant and anti-inflammatory activity against a wide variety of pathologies that are very topical in developed countries [139, 140,141] Recent studies have shown that lycopene reduces serum lipid levels, endothelial dysfunction, inflammation, blood pressure and increases antioxidant potential. [142] Likewise, it has been observed that these effects are significantly greater if a tomato paste (rich in lycopene) is mixed with extra virgin olive oil, the effect is significantly greater. These natural antioxidants, which can also improve the nutritional value of food, can lead to new forms if used in food... [143]. Cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality, and atherosclerosis is the common root of most CVDs. Oxidative stress is one of the most important factors driving atherosclerosis and its complications. [144,145]

On the other hand, several components, natural bioactives, such as polyphenols, have anticancer properties [146]. In a recent review, on the anticancer, antiprogressive and apoptotic effects of lycopene in prostate cancer. Observing the powerful effect that lycopene presents in this type of cancer[147].

In the process of aging, the dietary contribution of certain foods rich in antioxidants is reduced, due to different circumstances. In addition, the mitochondrial function is closely related to the processes of cell aging. If we manage to adapt a diet with combinations of certain foods rich in antioxidants, we will achieve healthier aging. And with lower risks of associated pathologies [148, 149].

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