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Oxidative stress markers: specificity and measurement techniques

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Summary. - With time, increasing evidence has been obtained of the involvement of oxidative stress in the pathophysiology of several diseases and ageing. Likewise, a large number of epidemiological studies have suggested that some pathologies can be prevented or delayed to some extent by dietary changes such as increased consumption of fruits, grains and vegetables. The above mentioned studies have suggested that the measurement of oxidative stress status, coupled to measurement of antioxidant status, may serve a role in diagnosis and/or treatment. The objective of this paper is to provide a review which, owing to the extent of the available literature, is obviously not exhaustive of current and most recent methods employed for the determination of the most specific markers of DNA, lipid and protein oxidative damage.

Key words: oxidative stress, biochemical markers, analytical methods.

Riassunto *(Specificità e metodi di analisi degli indici utilizzati per la valutazione dello stress ossidativo)*. - Nel corso degli anni è risultata sempre più evidente l'implicazione di fenomeni di stress ossidativo in numerose patologie e nell'invecchiamento. Inoltre numerosi studi epidemiologici hanno suggerito che alcune patologie possono essere prevenute o ritardate, in qualche misura, da modificazioni della dieta, quali un aumentato consumo di frutta, cereali e vegetali. Dagli studi sopra citati emerge che la misurazione dell'entità dello stress ossidativo insieme a quella della capacità di difesa antiossidante può risultare utile nella diagnosi e nel trattamento di alcune patologie. Scopo di questa rassegna è quello di fornire una raccolta dei metodi più recenti impiegati per la determinazione dei marker più specifici del danno ossidatico a carico di DNA, lipidi e proteine.

Parole chiave: stress ossidativo, marker biochimici, metodi analitici.

Introduction

During the years there is an increasing evidence for involvement of oxidative stress in the pathophysiology of several diseases and ageing [1-5]. For many of these pathologies oxidative stress is not necessarily the primary cause, but a secondary phenomenon no less important. For example, the oxidation damage of lipids in blood vessel walls seems to contribute to arteriosclerotic process. DNA oxidative damage may help tumor develop and accelerate ageing. An excessive production of free radicals is likely to be largely involved in tissue damage in rheumatoid arthritis and inflammatory diseases of the intestine such as Crohn's syndrome and ulcerative colitis. There is increasing evidence of oxidative damage in neurodegenerative diseases such as Parkinson's disease and brain trauma [1, 3-9]. Likewise a large number of epidemiological studies have suggested that some pathologies can be prevented or delayed to some extent by dietary changes such as increased consumption of fruits, grains and vegetables [1, 10-12]. This protective effect is reasonably ascribed to the high levels of dietary antioxidants (vitamin E, vitamin C, carotenoids, polyphenols, etc.) contained in this kind of food [1, 6]. Furthermore numerous nutrition intervention trials have been carried out on the ability of dietary antioxidants at nutritional or higher doses to prevent different diseases [10, 13].

The above mentioned studies have shown that oxidative stress increases in persons with certain diseases and that the measurement of an individual's oxidative stress status, joined with the measurement of his/her antioxidant status, may serve a role in diagnosis and/or treatment [13].

The measurement of the oxidative stress status requires reliable analytical methods for measuring both ongoing and steady-state (i.e. the balance between damage and repair) oxidative damage [2]. A variety of methods have been proposed in the literature; some of these techniques are non-invasive while others require the isolation of tissue fractions and can only be used in animal studies [2]. The objective of this paper is to provide a review, obviously not exhaustive owing to the extent of the available literature, of current and most recent methods employed for the determination of the most specific markers of oxidative damage.

Oxidative stress status measurement

Biological systems are continuously challenged with prooxidants that are either exogenously or endogenously generated. Our tissues are protected against this oxidative challenge by enzymatic and non-enzymatic antioxidants. What has come to be called oxidative stress occurs when the balance between prooxidants and antioxidants shifts in favour of prooxidants [2].

Cells often tolerate mild oxidative stress by up-regulating synthesis of antioxidant defense systems, however severe oxidative stress produces rises in intracellular free Ca^{2+} and iron, DNA damage, lipid peroxidation (membrane damage), protein oxidation and fragmentation (including membrane ions transporters), as well as carbohydrate damage. Cell injury and death may result [1, 14].

The first problem in the measurement of the oxidative stress status is to find suitable markers that are univocally able to detect and quantitate these phenomena.

The determination of species responsible for oxidative reactions, i.e. reactive oxygen species (ROS) and reactive nitrogen species (RNS), especially in free radical form, is very complex. There are many ways of studying free radicals, but all have to deal with the major problems associated with their high reactivity, namely their relatively short half-lives and migration distances. These features make the measurement of free-radicals very difficult [2]. The only technique which can detect radicals directly is electron spin resonance (ESR) spectroscopy [13]. Although the technique is highly sensitive, for practical reasons such method is limited to *in vitro* studies and to the determination of the identity of the free radical using the ESR spectrum [15, 16].

A more successful technique permitting *in vivo* ESR investigation of short-lived reactive free radicals by transforming them into more persistent species is the so-called "spin trapping" method [16]. The technique of spin trapping involves the addition of a primary free radical across the double bond of a diamagnetic compound (the spin trap) to form a radical adduct more stable than the primary free radical [17]. Similarly, free radicals can be trapped *in vivo* and detected *ex vivo* by their reaction with other chemicals such as salycilic acid. Finally, utilising the fact that reaction involving oxygen free radicals give rise to chemiluminescence, allows their reaction to be monitored both *in vitro* and *in vivo* in exposed organs [15].

Owing to the complexity of this type of analysis, detection and monitoring of free radical processes can be achieved only by detecting and measuring the products of their reactions with endogenous biocomponents. Specific products of such reactions or their metabolites may qualify as markers of a particular process or a specific free radical. In biosystems, these products are called molecular markers, which are a subclass of biomarkers. For a product to qualify as a molecular marker, there must be unequivocal proof of an exclusive origin to the product. First, a comprehensive understanding of the kinetics, energetics, and mechanisms of product generation is required. Second, other possible sources of the product must be excluded [18]. Virtually all possible targets of free radical attack can be measured, including the products of attack on nucleic acids, lipids, proteins, and carbohydrates [15].

DNA

The chemistry of DNA damage by several ROS and RNS has been well characterised *in vitro* although further studies are needed with some of them [19]. Neither O_2^- nor H_2O_2 under physiological conditions appear to cause strand breakage in DNA, or modification of bases in DNA [20, 21]. It has been proposed that much of the toxicity of O_2^- and H_2O_2 *in vivo* arises by their metal ion-dependent conversion into highly reactive hydroxyl radical (OH°) and/or other powerful oxidants [7, 20, 21]. To produce oxidative damage OH° must be made very close to the DNA since it is so reactive that it cannot diffuse from its site of formation [19, 22]. Thus, H_2O_2, which crosses biological membranes easily can be expected to penetrate to the nucleus and react with ions of iron or copper bound upon or very close to the DNA to form OH° [20]. This radical is so reactive that it can attack both the deoxyribose sugar and the purine and pyrimidine bases [7], and also produce cross links between DNA bases and nuclear proteins [20, 21]. OH° abstracts hydrogen atoms from deoxyribose, giving sugar radicals that can fragment in various ways releasing purine and pyrimidine bases and producing strand breaks [20]. Reactions of OH° with DNA bases are characterised by addition to the double bonds of these molecules to give adduct radicals of bases [18, 21]. In the presence of oxygen, pyrimidine radicals add oxygen to give corresponding peroxyl radicals [21]. By contrast, evidence indicates that the majority of purine adduct radicals do not react with oxygen. Subsequent reactions of base radicals lead to a variety of products from each of the DNA bases [21].

The situation with other reactive oxygen species is less clear-cut at present. Singlet oxygen is able to produce limited strand breakage in isolated DNA, and its ability to modify the DNA bases is also limited and appears selective for attack upon guanine [20]. Nitric oxide (NO°) and products derived from it (NO_2°, $ONOO^-$, N_2O_3, etc.) can cause nitrosation and deamination of amino group on DNA bases leading to point mutations [19, 22]. Deamination products of purine bases include xantine (from guanine) and hypoxantine (from adenine). 8-Nitroguanine may be a useful marker of attack on DNA by certain RNS such as $ONOO^-$ [22].

Peroxidizing lipids have been reported to damage DNA and also decompose to give a huge range of products including carbonyl compounds, such as malondialdehyde and 4-hydroxy-2-trans nonenal, which have been shown to be mutagenic to mammalian cells. If these aldehydes are generated in the vicinity of DNA, they may be able to combine with it to form distinctive products [20].

Another explanation of the ability of oxidative stress to cause DNA damage is that it triggers off a series of metabolic events within the cell that lead to the activation of nuclease enzymes, which cleave the DNA backbone without producing base modifications. These two mechanisms (DNA damage by ROS and RNS or by activation of nucleases) are not mutually exclusive, i.e. they could both take place. Indeed there is evidence consistent with both of them [20].

Oxidative DNA damage can be repaired by the action of a series of enzymes, but the existence of a low steady-state level of damage *in vivo* suggests that these enzymes may work close to capacity [19]. Nonspecific DNA repair enzymes excise DNA adducts to release deoxynucleotides, or specific DNA repair glycosylases release free bases. Deoxynucleotides are enzymatically hydrolyzed to deoxynucleosides, which are not usually further metabolized, and both these and free bases may be recovered in the urine [23]. Many modified DNA bases and nucleosides such as 8-hydroxyadenine, 7-methyl-8-hydroxyguanine, thymine glycol, thymidine glycol, hydroxymethyluracil, 8-hydroxyguanine, 8-hydroxydeoxyguanosine, 8-oxo-2'-deoxyguanosine and 8-oxoguanine have been detected in the urine of humans and other mammals [20, 22, 25]. The presence of these products in urine suggests that oxidative damage to the DNA bases does occur *in vivo* and that repair systems are active to cleave modified bases from DNA [20].

There are two types of measurement of oxidative DNA damage. Steady-state damage can be measured when DNA is isolated from human cells and tissues and analysed for base damage products: it presumably reflects the balance between damage and repair [19, 22]. Hence a rise in steady-state oxidative damage could be due to increased damage and/or decreased repair [22].

However, it is important also to have an index of total DNA damage [19] i.e. the "input" side of the steady-state equation. The most common approach has been to assess the "output" side, i.e., trying to estimate the rate of repair of oxidised DNA. Because accumulation of oxidative damage in DNA is limited, and the level of accumulation depends on the rate of free radical generation, the equilibrium is attained when: rate of damage = rate of repair [18]. Hence, under equilibrium conditions, the daily urinary output of markers represents the relative rate of damage due to oxidative stress [18]. However, it is possible that some excreted bases originate from diet or from the metabolism of the gut flora, and that DNA released from dead and dying cells within an organism undergoes rapid oxidative damage (since cell disruption can increase free radical reactions). Therefore, one must be cautious in using the amounts of modified DNA bases excreted from the body as an index of the extent of repair of oxidative DNA damage in healthy cells [20].

Different techniques admit sensitive detection, monitoring, and quantitation of DNA damage markers both locally in tissue and systemically in body fluids. Both approaches can be used to assess oxidative stress (Table 1) [18].

8-Hydroxy-deoxyguanosine (8-OHdG) and 8-hydroxyguanine (8-OHG) are the products most frequently measured as indicators of oxidative DNA damage [19, 20, 22, 23, 26-28].

The urinary excretion of the DNA repair product 8-OHdG has been proposed as a non-invasive biomarker of oxidative DNA damage in humans *in vivo* [23, 27]. The measurement of steady-state levels of an index like 8-OHdG in DNA combined with its urinary excretion rates, offer a powerful approach for estimating oxidative DNA damage and its repair [23, 28].

Table 1. - Principal markers of DNA oxidative damage

Markers	Techniques	Matrices	References
8-hydroxy-deoxy-guanosine	HPLC-ECD	Urine, DNA	[19, 23, 26]
8-hydroxy-guanine	GC-MS	Urine, DNA	[19, 21, 22]
8-oxo-2'-deoxy-guanosine	HPLC-ECD	Urine, DNA	[24, 25]
8-oxo-guanine	HPLC-ECD GC-MS	Urine, DNA DNA	[24, 25, 41] [31, 32]
5-(hydroxymethyl) uracil	GC	DNA, synthesized oligonucleotides	[34, 35]

Analysis of 8-OHdG using high performance liquid chromatographic (HPLC) coupled to electrochemical detection (ECD) is a highly sensitive technique that is frequently used after release of 8-OHdG from DNA in tissues, usually by enzymatic hydrolysis [19, 23], or from urine after solid-phase extraction procedure [26]. Levels of 8-OHdG in DNA increased with age in liver, and intestine but remained unchanged in brain and testes. The urinary excretion of 8-OHdG, which presumably reflects its repair from DNA by nuclease activity, decreased with age. These results suggest that the age-dependent accumulation of 8-OHdG residues observed in DNA from liver, kidney, and intestine is principally due to the slow loss of DNA nuclease activity; however, an increase in the rate of oxidative DNA damage cannot be ruled out [29]. Furthermore, since the 8-OHdG measured in the urine is likely to represent repair from all the cells of the body, it may not be possible to detect modest increases of damage occurring in a specific tissue or cell type because such damage would be diluted out by the high background level of 8-OHdG that is being excised continuously from other tissues [23]. Therefore, in order to detect measurable and significant differences in the levels of oxidative damage among individuals, the tissue whose DNA damage is being measured must represent a significant percentage of the whole body weight and the damage be elevated several-fold [23]. The level of 8-OHdG in urine is not affected by the diet since nucleosides are not absorbed from the gut [19]; however it is possible that some or all of the 8-OHdG excreted in urine may arise not from DNA, but from deoxyGTP (dGTP) in the DNA precursor pool of nucleotides. An enzyme has been described which hydrolyses dGTP containing oxidised guanine to prevent its incorporation into DNA. Because of these uncertainties, work is in progress to develop alternative urinary markers of total body oxidative damage [19, 22].

The gas chromatographic and mass spectrometry (GC-MS) technique has also been extensively used to study DNA base damage by various free radical-generation systems [21]. High sensitivity of detection can be achieved by operating the mass spectrometer in the selected ion monitoring (SIM) mode [20, 21]. GC-MS with SIM has been used to characterise oxidative DNA base damage by the identification of a spectrum of products, including 8-OHG, after acidic hydrolysis of DNA and derivatization (often by trimethylsilylation) to transform DNA bases and nucleosides into volatile products [19, 21, 22]. A mixture of endo- and exonucleases and alkaline phosphatase can also be used to hydrolyse DNA [21]. One advantage of the GC-MS approach is that measurement of a wide range of base damage products allows more accurate quantitation of DNA damage and can help to identify the ROS/RNS species that cause damage [12, 22].

However, the levels of 8-OHdG measured in DNA by HPLC/ECD are often less than the levels of 8-OHG measured by GC-MS/SIM. HPLC could understimate the real amount of 8-OHdG in DNA if the enzymatic hydrolysis is incomplete; the action of the exonucleases and endonucleases used to hydrolyse the DNA may be affected by the modification of the bases, and the acid pH often used for nuclease digestion could cause hydrolysis of 8-OHdG to 8-OHG, resulting in the loss of HPLC-detectable material [19, 22, 30]. By contrast GC-MS might overestimate 8-OHG as a result of their artifactual formation during the eating-step involved in classical silylation-based derivatization procedures [19, 22, 30]. Therefore any necessary heating stages should be done anoxically, also if it is difficult to remove O_2 completely [22].

HPLC with ECD is a highly sensitive and selective method for detecting also 8-oxo-2'-deoxyguanosine (oxo8dG) and 8-oxoguanine(oxo8Gua), other biomarkers of oxidative DNA damage [24, 25, 31], When employed together with the DNA isolation and monoclonal antibody-based immunoaffinity purification methods, oxo8dG and oxo8Gua in DNA and urine can be readily detected and quantitated, offering a powerful approach for assessing oxidative DNA damage *in vivo* [24]. Application of the technique to the detection of oxo8dG from DNA permits quantitation of the steady-state levels of this oxidatively modified deoxynucleoside, and overcomes the detection problems associated with the extremely low levels present in DNA [24]. Methods based on GC-MS have also been developed for the detection of 8-oxoGua in DNA. The background level of 8-oxoGua in DNA as determined by GC-MS is usually higher than that determined by the HPLC-ECD assay. The discrepancy between the two methods is due to an artifactual oxidation of guanine during the derivatization reaction as demonstrated by using pure guanine [31, 32]. The amount quantified by GC-MS is comparable to that obtained by HPLC-ECD when 8-oxoGua is prepurified by HPLC or by immunoaffinity chromatography, prior to the silylation reaction [31, 32].

An alternative approach to the measurement of oxidised bases makes use of repair endonucleases specific for certain kinds of lesion: endonuclease III, for oxidised pyrimidines, and formamidopyrimidine glycosylase for 8-oxo-Gua [32, 33]. These enzymes create breaks at sites of damage in DNA that are detected in numerous ways [32, 33]. Generally these methods give values lower than those obtained with GC-MS and HPLC [32, 33].

5-(Hydroxymethyl)uracil (HmU), derived from oxidation of the thymine methyl group, is another recognized endogenous DNA damage product, and HmU levels in DNA are increased by oxidant stress [34]. This hydroxylated base has beeen quantified in DNA by GC--MS using either acid or enzymatic hydrolysis of the DNA. Chemically synthesised oligonucleotides

Table 2. - Principal markers of lipids oxidative damage

Markers	Techniques	Matrices	References
Hydroperoxides	Enzymatic methods	Plasma	[41-43]
	Iodometric methods	Plasma, cellular membranes	[40, 44]
	HPLC-CL	Tissue, plasma, cellular membranes	[39, 47]
	HPLC-ECD	Plasma cells	[48, 49]
	GC-MS	Cellular membranes	[50, 51]
	HPLC-MS	Plasma	[52]
Conjugates dienes	Second derivative spectrophotometry	Plasma, tissue	[45, 46]
Total aldehydes	UV spectroscopy	Plasma, tissue	[53]
Malondialdehyde	TBA test	Plasma, serum, tissue	[36, 38, 55]
	HPLC	Plasma	[57, 58]
	GC-MS	Plasma, tissue, urine	[59]
4-hydroxynonenal	HPLC	Plasma, tissue	[53, 57]
	GC-MS	Plasma, tissue urine	[59]
Isoprostanes	GC-MS	Plasma, tissue, urine	[66-71]
	Immunoassay	Urine	[73, 75]
	Radioimmunoassay	Plasma, urine	[74]

containing known amounts of HmU as well as an isotopically enriched standard have been used to investigate the chemical modification of HmU during the acid hydrolysis of DNA and variations in derivatization efficiency [34, 35]. Both 5-(hydroxymethyl)uracile and thyamine were quantified in each DNA sample and the results expressed as a ratio. Quantitation of thymine was important due to possible variations in DNA hydrolysis efficiency for each sample [34]. Using enzymatic hydrolysis of the DNA the degradation of 5-(hydroxymethyl)uracile was avoided [35].

Lipids

Two general modes of lipid peroxidation may be defined, depending upon whether or not polyunsaturated fatty acids (PUFA) oxygenation is controlled enzymatically. Synthesis of a variety of lipid mediators, collectively termed eicosanoids, is dependent upon two types of oxygenases (cyclooxygenase and lipoxygenase) which catalize the introduction of molecular oxygen into non-esterified PUFA. The non-enzymatic, metabolic uncoupled peroxidation of PUFA/PUFA ester, also termed autoxidation, is stimulated by partially reduced oxygen and transition metals and by fatty hydroperoxides themselves [36]. Non-enzymatic peroxidation of tissue PUFA esterified to lipoprotein and biomembrane phospholipids appears an important manifestation of oxygen toxicity in biological systems [36, 37].

Lipid autoxidation is initiated by the attack of an acyl side-chain on a fatty acid by any chemical species that has sufficiently reactivity to abstract a hydrogen atom from a methylene carbon in the side-chain [37]. Fatty peroxyl radicals are early autoxidation intermediates, and fatty hydroperoxides are the primary initial oxidation products. Fatty hydroperoxides, howewer, are labile species and readily enter into radical reactions which lead to their molecular transformation and decomposition [36].

The extent of lipid peroxidation can be determined by a number of methods which can be divided between those that measure primary products of lipid peroxidation such as hydroperoxides, and those that measure the secondary breakdown products of lipid hydroperoxides such as secondary carbonyls (malondialdehyde, 4-hydroxynonenal) and volatile hydrocarbons (etane and pentane) [37].

The wide variety of purported analytical procedures for the evaluation of lipid peroxidation is perhaps most indicative of the fact that no single method sufficiently meets analytical standards in all applications (Table 2) [36].

Furthermore, the chemical composition of the end products of peroxidation will depend on the fatty acid composition of the lipid target and on what metal ions (if any) are free to partecipate in the reaction [37, 38]. So the selection of only a single test to monitor peroxidation can give misleading results [38].

For a most accurate measure of lipid peroxidation it is preferable to determine the primary products of oxidative attack, i.e. hydroperoxides, rather than their

secondary breackdown products or metabolites [37, 39]. In appropriate circumstances (i.e. where the decay of hydroperoxides is not significant) their measurement can be a useful index of early oxidative damage [40].

Several techniques based on different principles have been proposed to measure total lipid hydroperoxides such as enzymatic and iodometric methods, determination of conjugated dienes, etc.

Enzymatic methods are sensitive and can be quite specific but they are subject to interference by inhibitors and not all are stoichiometric. Modifications of the original method [41] had been recently proposed in order to eliminate interference and standardize the assay [42, 43].

The advantage of the iodometric hydroperoxide assay over other methods is the ability of hydroperoxide in a wide range of molecules to react with iodide quantitatively and with a known stoichiometry. Since many substances present in biological systems are known to interfere with this assay [40], modified methods have been proposed to correct interfering phenomena and provide more specific measurement [44].

It is generally accepted that the occurrence of conjugated dienes in lipids means autoxidation of lipids. The conjugated diene moiety of fatty acids hydroperoxides is a strong chromophore that can be detected spectrophotometrically. However, detection and quantitation of conjugated dienes is complicated by the end absorption exhibited by naturally occurring and non peroxidized lipids. Several methods have been proposed to circumvent this drawback, for example second derivative spectrophotometry, based on the differential of the first derivative spectrum [45, 46]. However, the presence of conjugated dienes does not necessarily imply that hydroperoxides are present. Thus, as a general suggestion, it is recommended that attention should be paid to interpreting their presence as a marker of ongoing lipid peroxidation in animal and human tissues, unless additional and more specific analyses are performed [46].

As regards these general methods more detailed information can be obtained using HPLC and GC techniques.

The HPLC technique allows the separation and quantification of a variety of products of lipid peroxidation such as free fatty acid hydroperoxides, cholesterol ester hydroperoxides, cholesterol hydroperoxides, phosphatidylcholine hydroperoxides [37]. Several HPLC methods have been proposed that use different systems for the detection of hydroperoxides.

The chemiluminescence-based high performance liquid chromatography (HPLC-CL) assay of lipid hydroperoxides is very sensitive and prevents the interference by biological antioxidants. This method has been utilized to detect cholesteryl ester hydroperoxide and phosphatidylcholine hydroperoxide in plasma and lipoproteines, and to separate membrane phospholipid hydroperoxides [39, 47].

A highly sensitive and simple method for the determination of cholesteryl ester hydroperoxides in plasma has been developed using high-performance liquid chromatography with coulometric electrochemical detection (HPLC-ECD) [48], and HPLC with reductive mode electrochemical detection on a mercury drop has been used for analysing lipid hydroperoxides in lipid extracts from leukemia cells [49].

Analysis of intact hydroperoxides by GC-MS has seldom been possible due to the thermal instability of these compounds. It was demonstrated that hydroperoxides can be converted to trimethylsilyl derivatives and analysed by GC-MS without significant thermal decomposition. The assay has been applied to the study of autoxidation of lipids in both *in vitro* and *in vivo* systems [50] and for quantitating low levels of hydroperoxides in biological matrices [51].

HPLC-MS was also used to determinate hydroperoxides; although this technique provides less resolution and lower sensitivity as compared to GC-MS, a distinct advantage was evident for direct measurements of some components [52].

However, the quantification of lipid hydroperoxides is, in practice, made difficult by the labile, fleeting nature of these species. Consequently, detection of lipid peroxidation has relied largely on the analyses of secondary or end products derived from hydroperoxide transformation, metabolism or decomposition [36].

Aldehydes are always produced when lipid hydroperoxides are metabolized in biological systems, and identification and quantification of these compounds gives an indirect index of oxidative injury which results in lipid peroxidation. Reaction with 2,4-dinitrophenil-hydrazine and the quantification by UV spectroscopy has been used as a measure of total aldehyde production [53]. This method lacks specificity and several techniques have been proposed to quantify specific aldehydes.

Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are frequently measured as indicators of lipid peroxidation and oxidative stress *in vivo* [54]. The determination of lipid hydroperoxides and MDA in addition to be used as an index of PUFA oxidation, takes on a greater significance with the publication of evidence implicating these products as secondary mediators of oxidative stress [37].

Also for 4-HNE the strong link between its biological activities and cellular damage indicates that its quantification may be a direct measure of an important toxic species [53].

Determination of MDA by thiobarbituric acid (TBA)-test is one of the most common assays used in lipid peroxidation studies [37]. The TBA test has no intrinsic specificity toward MDA; during the test many lipid-derived monofunctional aldehydes and several non-lipid biomolecules can directly interfere with spectrophotometric quantification [36, 38]. Most of the MDA detected

is generated by decomposition of lipid peroxides during the test, and great care is necessary to prevent artifactual oxidation of sample PUFAs [36, 38, 55]. Thus the TBA test does not measure only MDA formed in the peroxidation system and so TBARS (thiobarbituric acid reacting substances) seems to be a more appropriate term for this assay [38, 55]. Despite the problems that can occur with assays like the TBA test, it usually works adequately when applied to measurements of peroxidation in isolated membrane fractions such as liposomes and microsomes, or in precipitated lipoprotein fractions [37, 38, 55]. With biological materials, it appears prudent to consider a positive TBA-test response *per se* as are empirical indicator of the potential occurrence of peroxidative lipid injury rather than as a measure of lipid peroxidation [36].

To obtain more specific information several methods based on the HPLC analysis of free MDA, or MDA-TBA adduct have been developed [37, 55, 56].

The quantification of specific aldehydes of interest, such as MDA, 4-HNE, acetaldehyde, formaldehyde, propionaldehyde, has also been obtained using reversed phase HPLC separation of isolated 2,4-dinitrophenylhydrazine derivatives using a photodiode array or UV detector or by gas chromatography-mass spectrometry [53, 57, 58].

Another method used to quantitate saturated and unsaturated aldehydes, including MDA and 4-HNE, is based on the analysis of pentafluorobenzyl-oxime-trimethylsilyl derivatives by capillary gas chromatography-negative-ion chemical ionization mass spectrometry (GC/NICI-MS) [59].

Recently a method for the determination of MDA with capillary electrophoresis using UV detection also has been developed [60].

Besides these well recognized products of lipid peroxidation, there are a series of prostaglandin (PG)F_2-like compounds, termed F_2 isoprostanes, produced by the free radical catalysed peroxidation of arachidonic acid indipendent of the cyclooxygenase enzyme [61]. In addition to F_2 isoprostanes, it has recently been discovered that E-ring and D-ring isoprostanes are also produced *in vivo* [61, 62]

A substantial body of evidence has been obtained indicating that measurements of these unique products of lipids peroxidation provide a reliable marker of oxidant injury both *in vitro* and *in vivo* [61]. Measurement of isoprostanes esterified in tissue lipids can also be valuable in localising endogenous lipid peroxidation to a particular organ or tissue [62]. At least two of these compounds (8-iso-PGF_2, 8-iso-PGE_2) exhibit potent biological activity and thus they may not simply be markers of lipid peroxidation, but may also participate as mediators of oxidant injury [61, 62].

In addition to isoprostanes derived from arachidonic acid, peroxidation *in vitro* of eicosapentaenoic acid (EPA) gave rise to a family of F_3- isoprostanes [63], and peroxidation of docosahexaenoic acid (DHA) gave rise to a similar family of F_4-isoprostanes, called neuroprostanes [64]. In particular F_4-neuroprostanes were detected esterified in normal whole rat brain, in newborn pig cortex and in human cerebrospinal fluid. F_4-isoprostanes may provide a unique marker of oxidative injury to the brain and could potentially exert biological activity [65].

The methods used to detect and quantify isoprostanes are usually carried out using capillary gaschromatography/negative ion chemical ionization mass spectrometry (GC/NICI-MS) and most of them are developed to determine F_2-isoprostanes [66-71].

For the measurement of levels of isoprostanes esterified to tissue lipids, the isoprostanes must be hydrolyzed from tissue lipids either enzimatically or chemically, prior to quantification. Alkaline hydrolysis can be used to hydrolyze F_2- isoprostanes from phospholipids, whereas enzymatic hydrolysis must be used for D_2/E_2-isoprostanes because these compounds are readily dehydrated in the presence of a base [72].

While highly accurate, the mass spectrometric method of assay is labor intensive and the technology is not widely available. To overcome these problems immunoassay and radioimmunoassay methods for the measurement of 8-iso-PGF_2 have been developed [73-75].

Proteins

Damage to proteins may be initiated by ROS and RNS. ROS can be produced by a number of mechanisms, including irradiation, metal-catalized oxidation (MCO) systems, electron transport processes, and so forth. There is reason to believe that the most important mechanism is the action of MCO [76]. These systems are able to generate H_2O_2 and reduce Fe or Cu. The binding of reduced ions formed in these reactions to metal binding sites on proteins, followed by their reactions with H_2O_2, leads to the generation of OH°, which preferentially attacks amino acids residues at the metal binding site, leading in some cases to the transformation of side-chain amine groups into carbonyls [76-78]. It was demonstrated that the modification of proteins is initiated mainly by reactions with OH°; however the course of the oxidation processes is determined by the availability of O_2, $O_2^{-\circ}$ and HO_2°. Collectively these ROS can lead to oxidation of amino acid residue side chains, formation of protein-protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation [79-81]. The oxidative attack of the polipeptide backbone produces several categories of reactive species such as protein hydroperoxyde, protein-bound reductants notably 3,4-dihydroxyphenylalanine (DOPA) and aldehydes, and a range of stable products [82]. Peptide bond cleavage can also occur as a result of ROS attack by different pathways [79].

All aminoacid residues of proteins are susceptible to oxidation by OH°, but some of them are more susceptible than others. Cysteine and methionine residues are particularly sensitive to oxidation by almost all forms of ROS. Most biological systems contain specific reductases that can convert the oxidized forms back to their unmodified forms; these are the only oxidative modifications of proteins that can be repaired. Aromatic aminoacid residues are among the preferred targets for ROS attack. Tryptophan residues are readily oxidized to formylkynurenine and kynurenine and to various hydroxy derivatives; phenylalanine and tyrosine residues yield a number of hydroxy derivatives; histidine residues are converted to 2-oxo-histidine, asparagine, and aspartic acid residues [79]. Direct oxidation of lysine, arginine, proline, threonine and histidine residues may also yield carbonyl derivatives [77, 79]. In addition, carbonyl groups may introduce into proteins by reactions with aldehydes produced during lipid peroxidation or by glycation and glycoxidation reactions [79]. Among RNS the biological effects of peroxynitrite (PN) have been extensively studied. Methionine and cysteine residues are particularly vulnerable to oxidation by PN, and tyrosine and tryptophan residues are selective targets for PN-dependent nitration [79]. Rates of methionine oxidation and tyrosine nitration by PN are mutually exclusive processes, governed by the availability of CO_2, and under physiological conditions (in mammals), nitration will be largely, if not completely, favoured [76].

The intracellular level of oxidatively modified protein reflects the balance between the rate of protein oxidation and the rate of oxidized protein degradation. This balance is a complex function of numerous factors that lead to the generation of ROS, and that determine the levels of a multiplicity of enzymic and non-enzymic antioxidant defense systems, and the levels and activities of various proteases that degrade oxidized proteins [76, 79]. Changes due to radical damage, as well as other kinds of protein damage or abnormality, lead to accelerated catabolism and hence to the removal of damaged proteins from biological systems [81]. However, certain oxidized proteins are poorly handled by cells and, together with the possible alteration in the rate of production of oxidized proteins, this may contribute to the observed accumulation and damaging action of oxidized proteins during aging and in several pathologies [76, 81, 82].

The carbonyl content of proteins has been widely used as a convenient marker of oxidative protein damage under conditions of oxidative stress, and several sensitive methods for the detection and quantitation of protein carbonyl groups have been developed [76, 79, 83]. The appearance of such carbonyl groups is taken as presumptive evidence of protein oxidative modification because it is not entirely specific for this [77, 78, 84, 85]. For example, as mentioned above, glycation of proteins may add carbonyl groups to amino acid residues [78, 86]. In addition, there are other carbonyl groups existing in tissues that are not produced as a result of oxidation-mediated damage, and these are frequently not even proteins such as nucleic acids. Their presence in samples could lead to serious errors and overestimation of oxidation derived protein damage [83)].The carbonyl group has been measured using different techniques all of which are most suitable when using purified protein preparations; when working with non purified or crude extracts, serious limitations arise, which render interpretation of results difficult, even when the assay is coupled with HPLC gel filtration or electrophoresis for the removal of excess reagent [83], or with Western blotting for sensitive and specific detection [86].

Several approaches have been fallowed to detect and quantitate the levels of carbonyls in purified proteins as well as in protein of crude extracts of various tissues [77]. Among these, there are reaction with tritiated borohydride, reaction with 2,4-dinitrophenylhydrazine, and reaction with fluorescein thiosemicarbazide for gel electrophoresis [77, 78, 83]. Probably, the most convenient procedure to be used on a routine basis is the spectrophotometric method for the reaction of dinitrophenylhydrazine with protein carbonyls to form protein hydrazones [77].

As above mentioned, besides stable, relatively unreactive products of protein oxidation, such as protein carbonyls, radical attack on protein can generate two kinds of reactive moieties that are fairly long-lived under appropriate conditions: oxidising species such as protein hydroperoxides (oxidizing species) and reducing species such as protein-bound DOPA, formed by hydroxylation of tirosine [81, 87-90]. The long-lived nature of both the reactive moieties indicates that they may be able to diffuse and transfer damaging reactions to distant sites [81, 88, 89].

Like other hydroperoxides, protein hydroperoxides may contribute to oxidative stress by consuming cellular reductants, but they might be involved in local reactions on a protein surface resulting in further protein damage [88, 90]. In these reductions the major product is the hydroxide [90].

Determination of total protein hydroperoxides can be carried out using a iodometric assay, based on the spectrophotometric measurement of tri-iodide ion (I_3^-) formed during the test. The method is simple and relatively sensitive but has some disadvantages. For example, protein at a high concentration precipitates in the assay system and thus interferes with the accuracy [90-92].

The chemiluminescence-based HPLC assay widely used to study lipid hydroperoxides can be adapted for detecting also protein hydroperoxides. Hydroperoxy groups on different molecules have been found to respond differently in the chemiluminescence systems. Therefore, different hydroperoxides require different

calibration curves, which makes this method difficult to use for quantitative measurement of protein hydroperoxydes, especially in complex samples [90].

In the course of searching for a suitable marker for studying protein hydroperoxide, attention has been focused on the oxidation products of valine generated by free radical damage, because free valine is the amino acid on which hydroperoxides are most readily found during hydroxyl radical attack [87]. The fate of valine hydroperoxide in selected biological systems has been investigated by the use of chemiluminescence detection of hydroperoxides and HPLC analysis of *o*-phthaldialdehyde derivatives of amino acid residues [87]. The major degradation product of valine hydroperoxides was found to be valine hydroxides in several biochemical and cellular systems; this process possibly constituting a defence mechanism. It is therefore reasonable to choose valine hydroxides as markers for studying protein oxidation under oxidative stress [87]. The determination of both valine hydroperoxides and hydroxides requires protein hydrolysis as a necessary first step. The hydroperoxy group does not well tolerate the drastic conditions used for protein hydrolysis and is lost as unknown products, so a reduction step prior to hydrolysis is necessary to convert the hydroperoxy group into a hydroxy group using sodium borohydride [90].

It has recently been shown that, also leucine like valine, is oxidized to hydroperoxyleucines and their reduction give rise to hydroxyleucines. The occurrence of hydroxyleucines on proteins in physiological and pathological samples has been demonstrated by the HPLC technique. Thus hydroxyleucines, like hydroxyvalines, may provide useful *in vivo* markers for studying protein oxidation [93].

The reducing species, protein bound DOPA may also be of particular interest because of its ability to initiate secondary reactions, which again may result in the transfer of oxidative damage to the other targets and locations. To quantitate protein-bound DOPA a method by HPLC analysis with fluorimetric detection, after acid hydrolysis of protein, has been developed. Although it is fairly stable to acidic condition, care must be taken to avoid oxidative loss of DOPA during hydrolysis of the protein [90]. Another HPLC method used in conjunction of coulometric electrochemical array detection allows the determination of protein-bound DOPA and protein bound 3-nitrityrosine after enzymatic hydrolysis [94].

GC-MS of trifluoroethanol/pentafluoropropionic acid-derivatized compounds has been used to confirm the identification of the DOPA peak as well as to validate the quantification by HPLC [90].

Besides the above mentioned compounds other specific amino acid oxidation products have been proposed as markers of protein oxidative damage *in vivo*, namely such as 2-oxo-histidine, dytirosine, *o*-tyrosine, 3-nitrotyrosine, 5-hydroxy-2-amino valeric acid (HAVA).

Histidine is one of the amino acids most vulnerable to metal-catalysed free radical reactions. The conversion of histidine to asparagine has been established; however, asparagine may not be an appropriate marker for monitoring oxidatively modified proteins because it is detected as aspartate in the amino acid analysis following acid hydrolysis of oxidized proteins [95]. A procedure has been reported to monitor the oxidized histidine using HPLC with electrochemical detection that permitted the detection of the oxidized product with the highest specificity [95]. The main product of histidine oxidation was found to be identical to N-benzoyl-2-oxo-histidine; its acid hydrolysis provided a single product (2-oxo-histidine) that was sensitively detected by HPLC-ECD. Routine methods of amino acid analysis including ion exchange and reverse-phase chromatography have also been used to determine 2-oxo-histidine [96]. 2-Oxo-histidine has so far been detected in a number of proteins exposed to oxidative stress *in vitro*; thus it might be a useful probe to monitor oxidized protein. However, an assessment of the true physiological significance of the conversion of histidine to 2-oxo-histidine awaits extension of the study to mammalian sources [95].

The formation of dityrosine in a protein exposed to oxygen free radicals can serve as another marker of oxidatively modified proteins. Dityrosine release can be considered not only as a marker for protein oxidative damage, but also as an endogenous marker for selective degradation of oxidatively modified proteins [85]. Methods for assessing protein oxidative damage by measuring dityrosine formation and release, based on HPLC analysis with fluorescence detection have been described [85, 97].

In vitro studies have demonstrated that hydroxyl radicals are able to convert phenylalanine to tyrosine. When phenylalanine is irradiated with ultraviolet light, *p*-tyrosine, *m*-tyrosine and *o*-tyrosine are identified as hydroxylated products [98]. A sensitive method for the quantification of *m*-tyrosine and *o*-tyrosine has been described. The assay involves derivatization with pentafluorobenzyl bromide and extraction into *n*-decane, followed by GS/MS, and can be applied to the direct analysis of free amino acids or protein-bound amino acids following hydrolysis [99]. Others GC-MS methods employing N-propyl-heptafluorobutyryl derivatives have been proposed to quantitate *o*-tyrosine and 3-nitrotyrosine in tissues [100, 101]. As for the latter product, *in vitro* studies have shown that RNS derived from nitric oxide can generate 3-nitrotyrosine. Since *o*-tyrosine and 3-nitrotyrosine are stable to acid hydrolysis and not normally present in protein, they may serve as useful markers for oxidative damage [100]. To evaluate the potential usefulness of *o*-tyrosine and 3-nitrotyrosine as biomarkers *in vivo*, studies on protein oxidized *in vitro* has been carried out. *o*-Tyrosine is an excellent marker for protein damage *in vitro*, but it is not the major product

Table 3. - Principal markers of proteins oxidative damage

Markers	Techniques	Matrices	References
Carbonyl groups	Spectrophotometry	Tissue, serum, cerebrospinal fluid	[77]
Hydroperoxides	Iodometric methods HPLC	Tissue, cellular membranes Tissue	[90, 92] [90]
Valine hydroperoxides and hydroxides	HPCL	Plasma, cells	[87, 90]
Leucine hydroperoxides and hydroxides	HPLC	Tissue	[93]
Protein-bound DOPA	HPLC-FL HPLC-ECD	Tissue Tissue	[90] [93]
2-oxo-hystidine	HPLC-ECD Ion exchange chromatography	Experimental model Experimental model	[95] [96]
Dityrosine	HPLC-FL	Cells, cerebrospinal fluid, experimental model	[85, 97]
m-,*o*- tyrosine	GC-MS	Tissue	[99, 100, 101]
3-nitrotyrosine	GC-MS HPLC-UV HPLC-FL HPLC-ECD	Tissue Tissue, cells Plasma, tissue, cells Plasma peritoneal exudate	[101] [102] [102,103] [102, 104, 105]
5-hydroxy-2-animovaleric acid	GC-MS	Tissue	[83]

of phenylalanine oxidation by hydroxyl radical [100]. However it should be useful as a marker for hydroxyl radical-mediated damage *in vivo*. Studies of model proteins have demonstrated that 3-nitrotyrosine is a specific marker for protein oxidation by RNS and that this product should serve as an excellent marker for protein oxidized by RNS *in vivo* [100]. 3-Nitrotyrosine can be determined without derivatization by HPLC-UV detection, but this method is limited by its relative lack of sensitivity [102]. Sensitivity can be increased using fluorescence detection after derivatization of 3-nitrotyrosine [102, 103] or by electrochemical detection [102, 104, 105].

Oxidation of arginine and proline residues has been reported to produce (-glutamyl semialdehyde, which, on reduction and acid hydrolysis, form 5-hydroxy-2-amino valeric acid (HAVA) [83, 106]. Experiments utilizing purified preparations of amino acid homopolymers and purified proteins preparations in non-oxidized and oxidized states demonstrate that HAVA compares well with the carbonyl group formation and is a sensitive and specific marker of oxidized arginine and proline amino acid in protein [83]. HAVA can be determined using a GC-MS/SIM technique, and the amount of HAVA measured is a steady-state concentration dependent on its rate of formation and degradation under oxidative conditions. The rate of HAVA formation in each protein type will, therefore, depend on the amount of proline and arginine available to be oxidized as well as on the type and concentration of the oxidation species present [83]. However, the rate of HAVA lost *in vivo* will depend not only on oxidation conditions, but also on solvent properties and perhaps most importantly selective degradation of proteins containing oxidized altered aminoacids [83]. An advantage of this GC-MS/SIM technique is that additional products of oxidized amino acids such as dityrosine could potentially be detected [83].

The summary of principal markers of protein oxidative damage is shown in Table 3.

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