International Academic Research Journal of Internal Medicine & Public Health
ISSN Print : 2709-3301 | **ISSN Online :** 2709-331X
Frequency : Bi-Monthly
Language : English
Origin : Kenya
Website : https://www.iarconsortium.org/journal/iarjimph/home/

OPEN ACCESS JOURNALS

Review Article

Oxidative stress, antioxidants and biomarkers: appreciation for analytical methods for health promotion

Article History
Received: 10.06.2023
Revision: 15.06.2023
Accepted: 20.06.2023
Published: 26.06.2023

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How to Cite the Article:
Ana F. Vinha, *et al*., (2023 Oxidative stress, antioxidants and biomarkers: appreciation for analytical methods for health promotion. *IntAca. Res. J Int. Med. Pub. Hlth. 4*(3), 47-55

Abstract: Because of its extensive potential impact on human health, the determination of oxidative stress has been the main goal of study in several studies over the last few decades. The current work is a bibliographic review of the main methods of assessing oxidative stress in humans, taking into account the adverse effects it can do to individual quality of life and public health. To situate the theme, initially was described the concept of oxidative stress and the main diseases associated with it. Afterwards was presented the principal species of free radicals responsible for the oxidation, the antioxidant agents, enzymatic and non enzymatic , and the main bio marker of evaluation of oxidative stress. Regarding the analytical methods, it was proceeded by it's grouping according with the analyte in cause. In this regard of the reactive species of oxygen (ROS) and nitrogen (RNS), are presented the principal fluorescent and chemiluminescent probes, more concretely lucigenin, dihydroethidium DHE, and MitoSox, all characterized of the measurement of the superoxide, just like the relative to the determination of hydrogen peroxide as 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA), boronate and Amplex Red. About the RNS, are shown the 4,5-diaminofluorescein (DAF2-DA) and the dihydroethidium fluorescent probes as a method of determination of the level of NO and peroxynitrite, respectively. For biomarkers, the current work, presents the analysis methods dedicated to the detection of F2-isoprostanes, lipid hydroperoxides and malondialdehyde (MDA) associated with lipid peroxidation. Chromatographic methods, hyphenated with chemiluminescence spectroscopy, fluorescence, and mass spectrometry (MS/MS), and electrochemical detection (ECD) is the most frequently used along with the enzymatic immunoassays (ELISA). With regard to antioxidants, diversity methodologies is most evident, although the methods overridden by liquid chromatography.

Keywords: Oxidative stress, Reactive oxygen and nitrogen species; biomarkers; Antioxidants; Analytical procedures.

INTRODUCTION:-

Breafly, oxidative stress is caused by an imbalance in the generation and accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells and tissues, as well as the ability of cellular machinery to remove these by-products (Olufunmilayo *et al*., 2023). Free radicals and oxidative stress both contribute to aging and the development of several pathologies such as cardiovascular disease (Dubois-Deruy *et al*., 2020; Li *et al*., 2020; Hermida *et al*., 2018), chronic inflammatory diseases (Leyane *et al*., 2022; Cleeland *et al*., 2019), lung disease (Bezerra *et al*., 2023), diabetes (Sahakyan *et al*., 2022; Schwartz *et al*., 2016), depression (Ait Tayeb *et al*., 2023), cataracts (Kaur *et al*., 2012), neurodegenerative diseases such as Alzheimer's, Parkinson's (Olufunmilayo *et al*., 2023; Ionescu-Tucker & Cotman, 2021), Huntington's disease (Kumar & Ratan, 2016), amyotrophic lateral sclerosis (Motataianu *et al*., 2022), and cancers (Arfin *et al*., 2021; Jelic *et al*., 2021; Hayes *et al*., 2020).Indeed, the brain is particularly vulnerable to oxidative stress among the body's organs due to its high rate of oxygen utilization, weaker antioxidant enzymes, high content of easily oxidized polyunsaturated fatty acids, and terminal differentiation of neurons (Olufunmilayo *et al*., 2023). Oxygen free radicals, particularly the anion superoxide radical and the hydroxyl radical, are powerful initiators of lipid peroxidation, which is implicated in the etiology of a wide range of diseases (Martemucci *et al*., 2022). Taking into account the supposed role of oxidative stress in an extensive variety of diseases, antioxidants have become extremely popular as a means of preventing, improving, or curing a wide range of pathological situations. Some pathology management techniques, particularly neurodegenerative ones, have been centered on the creation of antioxidants as therapeutic and preventive agents. These include, among others, vitamin C, vitamin E, glutathione, coenzyme Q (CoQ), carotenoids, and melatonin. Moreover, a diet that includes fruits and vegetables,increases the antioxidant content in

human metabolism in addition to the synthesis of endogenous antioxidants. On the other hand,excess of antioxidant compoundsmight be harmful, as these chemicals can behave as pro-oxidants (Costa *et al*., 2018).

As far as we know, reactive oxigen species (ROS) play important and beneficial roles in the organism (Khan *et al*., 2023).ROS have been shown to be both damaging and beneficial in biological systems, depending on the environment. In low concentrations, for example, they contribute in detoxification events carried out by the cytochrome P-450 complex, pre-cancerous cells, and inflammatory processes, while also being beneficially related to signaling pathways that maintain cellular homeostasis in the body.

Among the reactive nitrogen species (RNS), the nitrogen monoxide radical ($^{\bullet}NO$), dinitrogen trioxide (N_2O_3), nitrous acid (HNO_2), nitrogen dioxide radical ($NO_2^{\bullet}$), nitrates ((NO_3^-) and peroxynitrite ($ONOO^-$) stand out. These species are involved in the regulation of apoptotic or necrotic cell death and are also recognized as important radicals.

Currently, measuring the degree of oxidative stress is not used in practice. Due to the lack of a standardized method for measuring the status of oxidative stress and lipid peroxidation in humans, clinic visits are limited. However, both the definition of oxidative stress as an imbalance between oxidants and antioxidants in favor of the antioxidants and the concept of redox disturbance by oxidants appear to need the measurement of oxidant generation vs. oxidant removal rates. These assessments are theoretically achievable, and a number of approaches have been developed in the hope of better understanding the role of oxidative stress in human diseases. It is critical to measure disease-associated oxidative stress in order to give health promotion.

2. Antioxidants

Antioxidant compounds have been extensively researched in biology, medicine, food, and nutrition sciences.The exposure of the organism to free radicals contributed to the development of defense systems to remove these free radicals. These defenses were evolution's response to the unavoidability of oxygen radicals in aerobic living environments (Kotha *et al*., 2022). Antioxidants are classified in a variety of ways, including (i) natural and synthetic; (ii) polar and non-polar; (iii) enzymatic and non-enzymatic; (iv) endogenous and exogenous; and (v) by the pathways in which they function (Mirónczuk-Chodakowska *et al*., 2018). Antioxidants function largely through three mechanisms: hydrogen atom transfer, single electron transfer, and metal chelation (Granato *et al*., 2018). They manifest their activity through three distinct channels: (i) preventive: preventing the production of free radicals and their products; (ii) disruption; interrupt radical oxidation reactions; and (iii) inactivation: inactivate free radical / radical derivative reactions products. Thus, an antioxidant is characterized as a chemical that can prevent or delay the oxidation of other molecules. In other words, they are chemicals or systems that can interact with free radicals safely and interrupt the chain reaction before critical molecules are damaged. These species can reduce the concentration of free radicals or prevent their generation (Martemucci *et al*., 2022), and antioxidants may neutralize the effects of reactive oxygen species under physiological conditions. Antioxidants act by acquiring or providing electrons to neutralize free radicals, thereby eliminating the unpaired radical state. As a result, antioxidant molecules can directly react with reactive radicals and destroy them, as well as change into new free radicals. However, these new radicals are less reactive, have a longer life, and are less dangerous than previously neutralized radicals. In other words, the antioxidant system acts as a kind of ROS scavenger. For example, superoxide dismutase (SOD) and catalase (CAT) convert H_2O_2 to $O_2^{\bullet-}$ and then to O_2. Several peroxide compounds, including hydrogen peroxide, can be inactivated by systems composed of glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase. In overall, antioxidants break the free radical chain by donating hydrogen, quenching atomic oxygen, dissolving peroxide, and blocking oxidative enzyme or UV radiation absorption (Ofoedu *et al*., 2021). The detrimental effects of ROS are balanced by the action of antioxidants, some of which are enzymes found in the body. Despite the presence of the cell's antioxidant defense mechanism to prevent oxidative damage from ROS, accumulating oxidative damage during the life cycle has been linked to aging and, in turn, to cardiovascular disease, cancer, neurodegenerative illnesses, and other diseases (Olufunmilayo *et al*., 2023; Leyane *et al*., 2022;Sahakyan *et al*., 2022; Dubois-Deruy *et al*., 2020).

Antioxidants may act at multiple phases of an oxidative radical process, as observed in the peroxidation of lipids in cell membranes, with an unavoidable activity in the subsequent stages, namely initiation, propagation, and chain termination. Reduced antioxidant levels produce reactive metabolites, and the body's antioxidant capacity declines with age (Kozakiewicz *et al*., 2019), making it vulnerable to adverse effects and providing poor protection. However, while antioxidants protect cells from detrimental effects, an excess of them might interfere with or even reduce the protective role (Costa *et al*., 2018). Aerobic metabolic cells have developed antioxidant defense systems that can be enzymatic or non-enzymatic, with the latter being exogenous or exogenous synthesis. Non-oxidant antioxidants, in addition to antioxidant enzymes, balance the detrimental effects of reactive oxygen species.

Antioxidants are classified into two groups: endogenous (enzymatic antioxidants), which include enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GPH-R), and glutathione peroxidase (GPH-Px), among others; and exogenous (non-enzymatic antioxidants), which include compounds such as glutathione (GSH),

vitamins A, C (ascorbic acid), and E (α-tocopherol), thiol antioxidants (glutathione, thioredoxin and lipoic acid) (Mirónczuk-Chodakowska *et al*., 2018), melatonin, carotenoids, natural flavonoids and polyphenols.Exogenous antioxidants available in food or nutritional supplements, such as ascorbic acid, tocopherols, phenolics and carotenoids, flavonoids and non-flavonoids, enhance the endogenous antioxidant defense system. Although some dietary components may not eliminate free radicals, they do promote endogenous activity and can thus be classed and labeled as antioxidants.Endogenous antioxidants provide an important role in sustaining normal cellular processes and, as a result, contribute to overall health and well-being. Endogenous antioxidants may not be sufficient in settings that induce oxidative stress, and dietary antioxidants may be necessary to maintain normal cellular activities (Kotha *et al*., 2022; Martemucci *et al*., 2022).

3. Biomarkers

Biomarker is a very broad term that can be used to describe any indicator of a biological state.A biomarker, also known as a biological marker, is defined as a "biological parameter susceptible to, or subject to, measurement and quantification (e.g., specific enzyme concentration, specific hormone concentration, specific gene phenotype distribution in a population, presence of biological substances), which serves as an indicator for health and physiology-related assessments."Ideally, but not always possible, a biomarker should have several characteristics, namely: specific and sensitive; stable, not susceptible to artifact-inducing or loss during storage; representative of the balance between oxidative damage production and clearance; determined by a specific, sensitive and reproducible method; to present a well-established relationship between the biomarker response and the induced damage, and to be a major product of the oxidative alteration that may be implicit in the development of the disease (Califf, 2018). Understanding the definitions for different biomarker classes while acquiring a better knowledge of their relevant applications could result in substantial advantages. Biomarker definitions established lately in a joint FDA-NIH website place different categories of biomarkers in the context of their respective functions in patient care, clinical research, or drug development. Complex composite biomarkers and digital biomarkers obtained from sensors and mobile technologies, as well as biomarker-driven prediction toxicology and systems pharmacology, are changing diagnostic and therapeutic technology development. Experimental, clinical and epidemiological studies are increasingly evaluating biomarkers of oxidative stress, such as CAT, CuZnSOD and malondialdehyde (MDA), due to their involvement in the pathogenesis of numerous diseases including atherosclerosis, cancer, diabetes, respiratory pathologies and among others. Indeed, these biomarkers play an important role in the pathogenesis or progression of many diseases (Vona *et al*., 2021).

Biomarkers are divided into three types: exposure, effect, and susceptibility.The function of an exposure biomarker is to identify exogenous substances in the system and is a product of the interaction between xenobiotic compounds and endogenous components related to exposure. In contrast, a biomarker of effect could be an indicator of an endogenous component of the biological system, a measure of the functional capacity of the system, or an altered state of the system that is recognized as disease. Finally, a susceptibility biomarker is defined as an indicator of the health of the system/organism sensitive to exposure to xenobiotic compounds. Due to their involvement in the pathogenesis of several diseases such as atherosclerosis, cancer, diabetes, respiratory pathologies, and others, oxidative stress biomarkers such as CAT, CuZnSOD, and malondialdehyde (MDA) are increasingly being studied in experimental, clinical, and epidemiological studies (Barbosa *et al*., 2021). Indeed, these indicators play an important role in the genesis or progression of many diseases.Among the many different markers, MDA and natural antioxidants, metalloenzymes, CuZnSOD, and GPx have been considered as the mostimportant stress biomarkers. However, measuring these molecules is difficult, due to the complexity of the biological matrices employed, particularly serum, blood, and urine, as well as a lack of correctly selective efficient extraction procedures which allow the simultaneous extraction of an extensive range of biomarkers.In fact, biomarkers can increase disease understanding and provide new knowledge about pathogenic pathways, allowing for earlier diagnosis and the use of more effective and safer medicines.

4. Oxidative stress detection methods

The evaluation and measurement of oxidative stress is far from being a simple and accessible process, whenever the techniques used for this purpose possess strong constraints that restrict throughout instrumentation and the reliability of results, respectively, due to difficulties in directly affecting the object of study and limitations of applicability. Direct analysis of reactive oxygen species in biological materials can be particularly difficult due to their high intrinsic reactivity and incredibly short half-life.

4.1. Reactive oxygen species determination methods

The detection approach for reactive oxidants differ only by whether they are inside or outside of the cell (Murphy *et al*., 2022). Intracellular detection is often accomplished using flow cytometry or fluorescence microscopy, whereas released oxidants can be identified in real time using fluorescence or UV/visible spectrometry, or by sampling the medium in ranges, as using HPLC or mass spectrometry. There are numerous ways for quantifying reactive oxygen species and nitrogen. Several approaches will be quoted in this paper, with an emphasis on the superoxide, hydrogen peroxide, nitrogen monoxide, and peroxynitrite species. According to some authors, the most convenient way to determine intracellular or intramitochondrial accumulation of ROS is the use of fluorescent or chemiluminescent assays

(Murphy *et al*., 2022; Akter *et al*., 2021). The probes used for this purpose are permeable cells and the measurements are usually based on the oxidative activity of ROS. Due to certain characteristics such as high sensitivity and ease of detection of small numbers of cells, chemiluminescence assays are widely used to detect reactive oxygen species. ROS-associated reactions involve multi-step radical mechanisms and have the same limitations as fluorescence assays.

Lucigenin

According to the scientific literature, the most frequently used chemiluminescent probeapplied to detect $O_2^{\bullet-}$ radicalis Lucigenin (bis-N-methylacridinium nitrate) becausethis has the characteristic of being permeable to the cell and is particularly specific. Likewise, this probe may have another nomenclature,namely chemiluminescent Lucigenin-enhanced. Briefly, the lucigenin probe is also considered a chemiluminescent, through a radical mechanism, despite the original compound being reduced to a radical that reacts with $O_2^{\bullet-}$to produce an intermediate that decomposes on emissionof light. That is, this reaction is effectively $O_2^{\bullet-}$ dependent.However, the validity of this technique has been questioned with the argument that $O_2^{\bullet-}$productioncan be artificially overestimated due to aphenomenon known as redox cycling, in which lucigenin radicals react with oxygen to generate $O_2^{\bullet-}$ (Lee *et al*., 2012). They likely react with a variety ofROS, and are therefore very unspecific.

Hydroethidine and and mito-hydroethidine

Hydroethidine (HE) probes and its derivative, mito-hydroethidine (mito-HE or mitoSOX red)are distinguished by their ability to detect the $O_2^{\bullet-}$ radical in cells and mitochondria.HE is modified to allow detection of $O_2^{\bullet-}$ in the mitochondria by adding a triphenylphosphonium group, which promotes its accumulation in the mitochondria. This modified analogue of HE is referred as MitoSOX, and has become commonly applied for the detection of $O_2^{\bullet-}$inside the mitochondria (Xiao & Meierhofer, 2019). The approach to HE has limitations because this molecule produces two luminous products with a red colour. The restriction of this approach is the generation of another oxidation product, luminous ethidium (E^+), which substantially adds to total fluorescence intensity, as well as overlapping fluorescence spectra. Thus, using fluorescence-based approaches to assess solely $O_2^{\bullet-}$can result in mistakes and inaccurate results.Additionally, it is necessary to confirm the formation of the E^+ using other analytical techniques such as: HPLC, by fluorescence or electrochemical detection, or LC-MS. The identical situation happens with MitoSOX, therefore it is likewise important to use HPLC technology to separate and identify the complex substance. Thus, the inability to detect other biological ROS such as H_2O_2 and $ONOO^-$ is a significant disadvantage. Furthermore, HE is vulnerable to autoxidation when exposed to light, so the experiment should be performed without light (Musakhanian *et al*., 2022).

2',7'-Dichlorodihydrofluorescein Diacetate

The 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probe is commonly applied to measure the level of intracellular ROS, particularly H_2O_2. In the same way, the applicability of this probe covers some RNS, especially intracellular NO and $ONOO^-$ (Kim & Xue, 2020). This technique is distinguished by DCFH-DA entering cells and concentrating primarily in the cytoplasm. Cellular esterases then cleave DCFH-DA to form 2',7' dichlorodihydrofluorescein ($DCFH_2$), which is further oxidized in the presence of H_2O_2, peroxidases, cytochrome c, and Fe^{2+} to form 2',7'-dichlorofluorescein (DCF).In this case, the fluorescence strength of DCF in the cytosol can be measured at 530 nm using a fluorescent plate reader when the sample is excited at 485 nm. A fluorescence at 530 nm can likewise be measured by a flow cytometer. Despite the popularity of this assay, however, it cannot be reliably used to measure intracellular H_2O_2 and other ROS for the following reasons: first - DCFH does not react directly with H_2O_2; second - various species of electrons oxidants oxidize DCFH to DCF; likewise, DCF can actually produce $O_2^{\bullet-}$and H_2O_2 through the reaction of the DCF radical with O_2, which can artificially increase the amounts of ROS that one is trying to quantify. Lastly, and not least, the transition metals, cytochrome C, and heme peroxidases can catalyze DCFH oxidation. As a result, using 2',7'-dichlorodihydrofluorescein diacetate probe is not the best method for quantify the H_2O_2 production (Ait Tayeb *et al*., 2023).

Boronates

Only a tiny number of small organic compounds react stoichiometrically with H_2O_2 to produce a detectable intracellular luminous result. However, boronates aromatics, specifically boronic acids and related esters, react with H_2O_2 to generate a single primary product, the corresponding phenol, with a high yield (Murphy *et al*., 2022). According toZhuang*et al*. (2017) a boronate moiety linked to a fluorophore, such as fluorescein, reacts with H_2O_2 to generate a highly luminous molecule. Boronate esters coupled to fluorophores are cell permeable and immediately interact with intracellular H_2O_2 to generate fluorescent products in cells.Although some of these probes have allowed for the detection and quantification of intracellular H_2O_2, such probe systems are limited in their applicability to biological systems due to their low selectivity, dependence on external enzymes, or incompatibility with biological settings. As a result, developing high-selectivity sensors to monitor H_2O_2 in biological processes is critical.According to multiple researchers, this approach is not restricted to H_2O_2, and that many additional ROS can be evaluated using this analytical technique. On the other hand,aromatic boronates may react stoichiometrically with $ONOO^-$over a million times faster than H_2O_2, which must be addressed when employing this analytical methodology.

Amplex® Red

Another probe that is often used to measure H_2O_2 is Amplex®Red, N-acetyl 3,7-dihydroxyphenoxazine (Noctor *et al*., 2015). The Amplex®Red is oxidized by horseradish peroxidase (HRP) and H_2O_2 to a fluorescent product, the resorufin. Although other single electron oxidizers are able to oxidize Amplex®Red to resorufin, oxidation catalyzed by HRP in the presence of H_2O_2 is highly efficient and greatly increases the yield of resorufin (Zhao*et al*., 2012). Therefore, this test is a viable method to continuously measure the formationextracellular H_2O_2 (Noctor *et al*., 2015). An important complicating factor is the lightmediated by the photochemical oxidation of resorufin in the presence of biological reductants,such as glutathione and NADH, which induce the formation of radicals mediated byresorufin, providing some drawbacks due to radical reactions induced by resorufin. In summary, when H_2O_2 is released from the cell or in mitochondrial cell preparations, the Amplex®Red probe can be used to quantify H_2O_2 in the presence of HRP.

4.2. Fluorescent and chemiluminescent probes for RNS determination

In the scientific literature, there are numerous probes for the detection of ROS and, likewise, several studies have described the application of fluorescent and chemiluminescent probes for the determination of RNS.In this article, we will focus on 4,5-Diaminofluorescein diacetate (DAF2-DA) and Dihydrorhodamine (DHR) probes, which are used to determine $NO^{\bullet}$ and $ONOO^{-}$, respectively (Zielonka *et al*., 2012). There are now multiple fluorescence-based approaches for detecting $ONOO^{-}$ that have been developed by various investigations. They are, however, not frequently utilized anymore.

Diacetate of 4,5-Diaminofluorescein

The 4,5-Diaminofluorescein diacetate probe (DAF2-DA) is a fluorescent technique used to measure $NO^{\bullet}$ (Li *et al*., 2013). DAF2-DA interacts with $NO^{\bullet}$ in the presence of O_2 in cells to produce the highly fluorescent triazole fluorescein (DAF-2T), which can be fluorometrically detected at 538 nm and has a green color when stimulated at 485 nm. The DAF2-DA technique, due to its high cellular permeability, enables the monitoring of dynamic fluorescence changes in cells, which is a frequent property of fluorescent probes (Zhao*et al*., 2012).

The fluorescence of dihydrorhodamine

Dihydrorhodamine fluorescence (DHR) is the most commonly used probe for monitoring reactive nitrogen species, specifically $ONOO^{-}$(Kalyanaraman *et al*., 2012). It is distinguished by the fact that it is a luminous red dye (Zhao*et al*., 2012). DHR is first oxidized to rhodamine 123, a lipophilic and positively charged molecule, and then the membrane potential traps it in the mitochondria. When the sample is stimulated at 500 nm, the accumulated rhodamine 123 is detected around 536 nm. The oxidative conversion of DHR to rhodamine is mediated by a DHR intermediate radical which, however, can be reduced by thiols and ascorbic acid. Consequently, it leads to false-negative results. Therefore, DHR can only be applied as a non-specific intracellular indicator of $ONOO^{-}$ and HOCl (hypochlorous acid) formation.

5. Biomarker determination methods

The biomarker values in the analysis of pooled data from several studies containing a biomarker exposure can vary among laboratories and normally require calibration to a reference assay prior to pooling. There is a wide range of oxidative stress biomarkers and laboratory techniques available, each of which has its own advantages and limitations (Cheng & Wang, 2020). Biomarkers are required to measure the state of oxidative stress in vivo, for early detection of diseases or their progression, and, in particular, to assess the efficacy of pharmacological or antioxidant treatment (Vona *et al*., 2021). Because of their inherent properties, quantifying free radicals created in vivo is a complex operation. As a result, chemicals associated with lipid and protein peroxidation have been frequently used to measure oxidative stress. Among the different existing markers, the F2-IsoPs, 8-iso-prostaglandin F2α, lipid hydroperoxides, MDA, 8-OHdG, 8-OHG/8oxoGuo, are currently essential for the determination of oxidative stress.

F2-IsoPs, prostaglandin-like compounds formed from the free radical-mediated oxidation of arachidonic acid , are the ‘gold standard’ for measuring oxidative stress in the body. F2-IsoPs also have potent biological effects associated with inflammation and therefore may mediate chronic disease initiation and progression (Vona *et al*., 2021). Additionally, F2-IsoPs may also act as potent vasoconstrictors via thromboxane formation in the endothelium, and promote platelet activation resulting in thrombus formation. Accoding to Sampson *et al.* (2002), 8-iso-prostaglandin-F2α (8-iso-PGF2α), is one of the stable products of non-cyclooxygenase peroxidation of arachidonic acid, has proved to be the most available and reliable marker of lipid peroxidation *in vivo, and it appears to be more sensitive and selective than other oxidative stress markers.* Lipid peroxidation is a complex process known to occur in both plants and animals. It involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids and the eventual destruction of membrane lipids, with the production of a variety of breakdown products, including alcohols, ketones, alkanes, aldehydes and ethers. Lipid hydroxides such as hydroxyoctadecadienoic acids (HODE), hydroxyeicosatetraenoic acids (HETE), and hydroxycholesterols may be recommended as reliable biomarkers. Notably, the four HODEs, 9-*cis*,*trans*, 9-*trans*,*trans*, 13-*cis*,*trans*, and 13-*trans*,*trans*-HODE, can be measured separately by LC–MS/MS and the *trans,trans*-forms are specific marker of free radical mediated lipid peroxidation. Further, isoprostanes and neuroprostanes are useful biomarker of lipid peroxidation (Ito *et al*., 2019).

Regarding, 8-hydroxy-2'-deoxyguanosine (8-OHdG) or 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), this biomarker has been commonly used as biomarker of oxidative stress. However, currently it is currently used in the diagnosis of mental illnesses, including schizophrenia, bipolar disorder and depression (Goh *et al*., 2021). The molecule 8-OHdG is the most mutagenic as a result of oxidative stress and can be tested independently. HPLC, GC/MS, immunohistochemistry, and ELISA techniques were established to quantify 8-oxoGua and 8-oxodG bases in human DNA samples. The specificity of the ELISA approach has been questioned, and it has been confirmed that using this test results in an overestimation of the yield of 8-oxodGuo when compared to HPLC-MS/MS data. However, to date, there is no consensus on the most suitable biomarkers of oxidative stress in general. Liquid chromatography (LC) is an available analytical procedure that provide higher sensitivity and specificity. UV/visible spectroscopy, HPLC-CL, HPLC-FL, HPLC-ECD, and LC-MS/MS are all possible post-column detection methods.

6. Determination of antioxidants

Antioxidant defenses are composed of several separate chemical constituents, making simultaneous quantification not possible. Furthermore, antioxidant levels alone do not reflect defense strength. The levels of antioxidants, vitamins, or antioxidant enzymes are just instructive; they only reflect one aspect of redox equilibrium, leaving the question unanswered as to whether a drop in levels is also symptomatic of increased oxidative damage.The primary exogenous and endogenous antioxidants will be reviewed in this sub-chapter in order to maintain consistency of information when compared to biomarkers.

Ascorbic acid

There are many methods for the determination of ascorbic acid in various biological samples. Titration with a dichlorophenol indophenol oxidant solution (DCPIP), potassium iodate, or bromate is a traditional method of assessing ascorbic acid. Analytical methods for determining this antioxidant include conventional titration, fluorometric spectrometry, chemiluminescence, capillary electrophoresis, electrochemical methods, amperometric methods, enzymatic methods, and HPLC methods. Similarly, ascorbic acid can be spectrophotometrically analyzed based on its reaction with hexacyanoferrate(III), oxidation utilizing the Cu(II)-neocuproine complex, or measurement of iodine that reacts with ascorbic acid. Cheiluminescence is another optical approach for measuring vitamin C. In this context, an enzymatic approach based on a highly specific ascorbate oxidase (EC 1.10.3.3) can be employed to determine ascorbic acid levels. Regarding chromatographic methods, particularly HPLC-UV/DAD has been applied in the evaluation of ascorbic acid in food products (Gîrd, 2018) and in biological fluids (Azmi *et al*., 2022). Using the HPLC technique with UV detection it is, probably the simplest and most cost-effective approach for measuring ascorbic acid in biological materials (Azmi *et al*., 2022). Because of the natural oxidation process of ascorbic acid, sample preparation is the most difficult challenge for its determination. Temperature, light, pH, the amount of oxygen dissolved in the sample, the solvent employed, the ionic strength, and the presence of enzymes, oxidants, and certain divalent cations all have an impact on ascorbic acid oxidation (Salkic & Selimovic, 2015). The HPLC methodology with electrochemical detection is an efficient technique for measuring ascorbic acid in food products and biological fluids, like intracellular ascorbic acid levels in human peripheral blood mononuclear cells and in plasma, due to its simplicity, low cost, selectivity and sensitivity (van Gorkom *et al*., 2022).

α- tocopherol

The reversed-phase HPLC method is the chosen methodology in the majority of the published literature to date. However, technologies such as fluorometric, electrochemical, mass spectrometry, and UV detectors have also been used (Słowik-Borowiec *et al*., 2023). However, different types of biological matrices necessitate distinct α-tocopherol analysis approaches. In general, reversed phase HPLC with a wide range of mobile phase compositions is used to assess plasma α-tocopherol concentration. One of the key issues in the measurement of α-tocopherol is that the purification and detection techniques in chromatography varied for each form. As a result, it is difficult to select a single method to detect α-tocopherol in samples that include multiple types, such as plasma, erythrocytes, cultured cells, or cellular tissues (Cuerq *et al*., 2016).

Glutathione and Glutathione Disulfide

Glutathione, apart from being an important antioxidant, is the main low-molecular-weight thiol-containing peptide with its cysteine residue present in most living cells.Because of glutathione (GSH)'s vital involvement in the process of protection against oxidative stress, there has been an increase in interest in determining GSH and glutathione disulfide (GSSG). GSH levels are often measured in whole blood, plasma, and erythrocyte hemolysate, as well as directly on tissue (Alisik *et al*., 2019). Several approaches for determining GSH have been suggested so far. Several methods stand out among the others: spectrophotometric, fluorimetric, electrochemical, chemiluminescent, and nuclear magnetic resonance (Gaucher *et al*., 2018). Likewise, separation methods such as high performance liquid chromatography (HPLC), gas chromatography (GS) and capillary electrophoresis have been developed. Chromatographic methods employing various detectors, such as UV-Vis, fluorescence, capillary electrophoresis, and MS mass spectrometry, are now the most appropriate (Wang *et al*., 2019). However, these methods often lack sensitivity and selectivity and may require time-consuming pretreatment of the sample, such as derivatization with fluorescent reagents. The advantage of

utilizing HPLC with electrochemical detection, employing either amperometric or coulometric electrodes, is that GSH and GSSG can be measured directly. These methods, together with HPLC/MS spectrometry assays, circumvent the common issues associated with derivation procedures (Cheng & Wang, 2020; Duhamel *et al*., 2019).

The GSSG/GSH ratio is regarded as an essential indication of cellular oxidative stress (Nuhu *et al*., 2020). Normal GSSG concentrations are substantially lower than GSH concentrations, necessitating very accurate and exact GSSG determination in the presence of such high GSH concentrations. Furthermore, GSH depletion is frequently used as a measure of oxidative stress. Another alternative method to determine GSH levels is through fluorescent probes for glutathione. Under certain circumstances, such as at the onset of apoptosis, glutathione may efflux from cells, which reduces the reducing capacity of the cells and therefore may result in oxidative stress without interfering with ROS production (Peoples *et al*., 2019).

7. CONCLUSIONS

In the beginning, scientists were interested in reactive oxygen species because of the pathology associated with the negative effects of aerobic respiration, a necessary evil caused by leakage from the electron transport chain in mitochondria.The role of these substances in aging, chronic illnesses, and cancer was studied in this context.The finding that the oxidative burst by phagocytic cells was caused by the purposeful generation of reactive oxygen species opened up a new area. Because the level of damage to be evaluated is generally low, measuring reactive oxygen and nitrogen species and determining the accompanying oxidative damage in living organisms is a difficult process. Due to its higher sensitivity, the HPLC-MS/MS approach through electrospray ionization is currently the recommended method among the various technologies offered to quantify these lesions.Antioxidant defenses, on the other hand, comprise a wide range of natural substances; ascorbic acid, vitamin E, and glutathione (GSH), glutathione disulfide (GSSG) have been highlighted across this article. The HPLC-ECD technique is the most commonly used to identify ascorbic acid in biological fluids. In terms of -tocopherol measurement, the HPLC-PDA approach, in contrast to the difficulty displayed by other techniques, was successfully employed in multiple biological materials. Finally, HPLC-FL or LC MS/MS are advised to measure GSH and GSSG. With regard to oxidative stress, new methodologies or improvements to known methodologies are referenced every year. The continuation of research suggests that the main limitations, particularly those relating to the distinction between the normal oxidative process and oxidative stress, will be overcome in the near future, namely with regard to the typical biomarkers of each process and the sensitivity of their detection.

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