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Acta Medica Marisiensis 2016;62(1):112-120 DOI: 10.1515/amma-2015-0070

UPDATE

Is the Oxidative Stress Really a Disease?

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Oxidative stress is an imbalance between free radicals or other reactive species and the antioxidant activity of the organism. Oxidative stress can induce several illnesses such as cardiovascular disease, neurodegenerative disorders, diabetes, cancer, Alzheimer and Parkinson. The biomarkers of oxidative stress are used to test oxidative injury of biomolecules. The indicators of lipid peroxidation (malondialdehyde, 4-hydroxy-2-nonenal, 2-propenal, isoprostanes), of protein oxidation (carbonylated proteins, tyrosine derivatives), of oxidative damage of DNA, and other biomarkers (glutathione level, metallothioneins, myeloperoxidase activity) are the most used oxidative stress markers. Diseases caused by oxidative stress can be prevented with antioxidants. In human body are several enzymes with antioxidant capacity (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase) and spin traps. Antioxidants are synthetized in the organism (glutathione) or arrive in the body by nutrition (ascorbic acid, vitamin E, carotenoids, flavonoids, resveratrol, xanthones). Different therapeutic strategies to reduce oxidative stress with the use of synthetic molecules such as nitrone-based antioxidants (phenyl-α-tert-butyl-nitrone (PBN), 2,4-disulphophenyl-N-tert-butylnitrone (NXY-059), stilbazulenyl nitrone (STAZN), which scavenge a wide variety of free radical species, increase endogenous antioxidant levels and inhibits free radical generation are also tested in animal models.

Keywords: free radicals, biomarkers of oxidative stress, antioxidant enzymes, resveratrol, nitronebased antioxidants

Received: 30 March 2015 / Accepted: 04 June 2015

Introduction

Oxygen, an important element for the organisms, in certain conditions can induce several illnesses in humans such as cardiovascular disease, neurodegenerative disorders, Alzheimer, Parkinson, diabetes, cancer, liver cirrhosis, obesity and metabolic syndrome. During mitochondrial respiration, electrons are given in steps to oxygen, process that leads to the formation of ROS (reactive oxygen species) and secondary to RNS (reactive nitrogen species) [1]. Oxygen is the ultimate electron acceptor in the electron flow system that yields energy. Problems can occur when the electron flow becomes uncoupled, generating free radicals [2].

Numerous enzymes are present in the human body and function as promoters of the reactive species generation, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, nitric oxide synthase (NOS), myeloperoxidase (MPO), lipoxygenase (LO), xanthine oxidase (XO). As a compensatory mechanism, several antioxidant enzymes are present, which have the ability to capture or neutralize free radicals, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase, glutathione transferase, oxidoreductase, thiol-disulfide and peroxiredoxins [3].

In human body a pro-oxidant-antioxidant balance exists. When the production of reactive species is increased significantly, or when the levels of antioxidants are reduced, a new pathological state (the oxidative stress) appears [2].

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1. Free radicals and other reactive species involved in the oxidative stress

Free radicals are highly unstable molecules in biological systems that have one or more unmatched electrons available to react with lipids, enzymes, proteins and DNA. Non-radical reactive species do not have unpaired electrons but are able to oxidize biomolecules. The reactive species are classified to:

a.) Free-radical species: superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($HO^\bullet$), hydroperoxyl radical ($HOO^\bullet$), lipid radical ($L^\bullet$), lipid peroxyl radical ($LOO^\bullet$), peroxyl radical ($ROO^\bullet$), lipid alkoxyl radical ($LO^\bullet$), singlet oxygen ($^1O_2$), nitrogen dioxide (NO_2), nitric oxide (NO), thiyl radical ($RS^\bullet$), protein radical ($P^\bullet$).

b.) Non-free radical reactive species: hydrogen peroxide (H_2O_2), ozone (O_3), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), nitrous acid (HNO_2), peroxynitrous acid (ONOOH), nitryl chloride (NO_2Cl), nitrous oxide (N_2O), nitroxyl anion ($NO^\bullet$), lipid hydroperoxide (LOOH).

For normal cell function the ROS synthesis at physiological concentration is absolutely necessary. Phagocytic cells produce free-radicals, such as $O_2^{\bullet-}$ or NO, in order to destroy foreign organisms. If ROS are produced in high amounts, they can stimulate free-radical chain reactions that damage proteins, lipids, and nucleic acids and finally cause disease conditions [2].

1.1. Superoxide radical anion ($O_2^{\bullet-}$)

Superoxide anion is the main free radical produced at high rates in human organisms by the univalent reduction of molecular oxygen during normal cellular metabolism [4]. $O_2^{\bullet-}$ arises from multiple sources: mitochondria electron

transport chain (oxidative phosphorylation), NADPH oxidase, XO, etc. [5]. $O_2^{\bullet-}$ are further converted into H_2O_2 predominantly by SOD [6].

The aerobe organisms obtain energy through oxidative phosphorylation, which occurs in the mitochondria. By oxidative phosphorylation adenosine triphosphate (ATP) is formed, while electrons are transferred from adenine dinucleotide (NADH) or flavin adenine dinucleotide ($FADH_2$) to molecular oxygen. This relocation occurs through the electron transport chain (ETC) localized in the inner mitochondrial membrane. Under pathological conditions oxidative phosphorylation can be inaccurate and results in the generation of $O2^{\bullet-}$.

The family of NAD(P)H oxidases (Nox) is an important membrane-bound enzyme complexes, source of $O_2^{\bullet-}$ generation, through electrons transfer from NADPH to oxygen. Seven isoforms of the NADPH oxidases are identified in mammalian cells: Nox1 to Nox5, Duox1 and Duox2. Nox2 is present moustly in the phagosomal membrane, wich reduce oxygen to superoxid. The microbicial hypohalous acids play an important role in the immune system [7]. Nox4 is the major isoform expressed in adipocytes, it primarily generates H_2O_2, then other NADPH oxidases would further generate $O_2^{\bullet-}$ [3, 6].

Xanthine oxidase (XO) is an iron sulfur molybdenum flavoprotein present in the endothelial cells and in plasma but not in smooth muscle cells. It generates $O_2^{\bullet-}$ through catalyzing the hypoxanthine transformation to xanthine, after than xanthine conversion to uric acid. In several studies a negative correlation between XO endothelial activity and vitamin C effects was shown. The patients with coronary artery disease also show an increased XO activity [3].

Another important biologically activity of anion superoxide is its cytotoxic activity used by the immune system to kill invading microorganisms.

Due to its capacity to generate other free radicals, superoxide anion can cause cell injuries. Its mechanism of toxicity involves iron release from the iron-sulfur clusters in proteins, affecting iron containing enzyme functions. $O_2^{\bullet-}$ reacts with other reactive species and can generate more destructive species, such as:

- peroxynitrite ($ONOO^-$)
- hydroxyl radical ($HO^\bullet$) [8].

1.2. Hydroxyl radical ($HO^\bullet$)

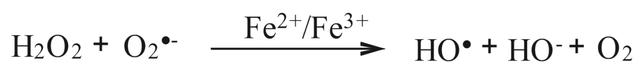
Hydroxyl radical is known as the most reactive specie, with an estimated half-life of about 10^{-9} s. It can be formed from water through high-energy irradiation or from H_2O_2 in metal-catalyzed Fenton reaction (Figure 1) [2].

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^\bullet + HO^-$$

$$Fe^{3+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2$$

Fig. 1. Fenton reaction

The two steps of Fenton reaction can be described whith the Haber-Weiss corelation (Figure 2) [9].



$$H_2O_2 + O_2^{\bullet-} \xrightarrow{Fe^{2+}/Fe^{3+}} HO^\bullet + HO^- + O_2$$

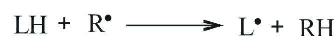
Fig. 2. Haber-Weiss reaction

A special case of the Fenton reaction is the generation of the hydroxyl radical from the hydroxyl group of water, when one electron is transferred to the ferric ion with the formation of a divalent iron [10].

Numerous metal ions in their lower oxidation states such as Cu^+, Ti^{3+}, Cr^{2+}, Co^{2+}, etc, react with H_2O_2 in a similar pattern as Fe^{2+}, and the reactions of these metals with H_2O_2 are thus called "Fenton-like" reagents. The reduction of the concentration of ferrous and other metal ions may manage protection against oxidative stress [2].

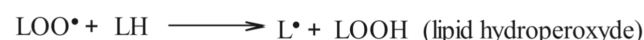
The most important negative effect of hydroxyl radical is the involvement in the lipid peroxidation. $HO^\bullet$ radical can start a chain reaction, a transition metal-catalyzed reaction, with the formation of lipid hydroperoxide and another free radicals, through carbon-centered radical ($LOO^\bullet$) formation (Figure 3) [3].

$$LH + HO^\bullet \longrightarrow L^\bullet + H_2O$$



$$LH + R^\bullet \longrightarrow L^\bullet + RH$$

$$L^\bullet + O_2 \longrightarrow LOO^\bullet \text{ (lipid peroxyl radical)}$$



$$LOO^\bullet + LH \longrightarrow L^\bullet + LOOH \text{ (lipid hydroperoxyde)}$$

Fig. 3. Chain reaction of lipid peroxidation

The damage of DNA bases can also be attributed to the hydroxyl radical, DNA oxidation being another cellular damage induced by oxygen radical species [11]. In the presence of hydroxyl radical, fibrinogen (the most hydrophobic protein in circulation) undergoes a drastic structural change, forming an insoluble fibrin-like precipitate. The quantity of precipitated fibrinogen is equivalent to the amount of the hydroxyl radical formed in the system [10].

1.3. Singlet oxygen ($^1O_2$)

Singlet oxygen is an excited state of molecular oxygen. It is less stable than the triplet oxygen. Its half-life (~10^{-6}s) depends on the nature of the neighboring medium. It binds preferentially to the double bonds in the polyunsaturated fatty acids or in the guanine in DNA bases, forming endoperoxides that can be reduced to alkoxyl radicals that initiate radical chain reactions. Singlet oxygen is responsible for UV radiation skin damage, cataract formation, macular degeneration [2].

1.4. Peroxyl- ($ROO^\bullet$), lipid peroxyl- ($LOO^\bullet$), and alkoxyl radical ($RO^\bullet$)

Peroxyl- and lipid peroxyl radicals have relatively long half-lives, having time for diffusion in the biological systems.

They are generated by the lipid peroxidation, which is started by the abstraction of an H atom from the polyunsaturated fatty acids, initialized by the hydroxyl radical [2, 12].

Lipids can be oxidized by the different reactive species through LO enzyme catalysis. It catalyzes the stereospecific insertion of molecular oxygen into polyunsaturated fatty acids to give rise to a complex family of biologically active lipids: carbon-centered lipid radical ($L^\bullet$), lipid peroxyl radical ($LOO^\bullet$), lipid hydroperoxide (LOOH) [3, 11].

Products of lipid peroxidation are peroxyl radical and organic hydroperoxide (LOOH or ROOH). They can reorganize to endoperoxide which are cleaved to yield aldehydes, such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 2-propenal (acrolein) and isoprostanes as terminal products, which also cause tissue damage [8]. These aldehydes can react with amine groups of proteins modifying the structures of lipoproteins. They are also toxic and active mutagens [2, 11].

1.5. Nitric oxide (NO)

Nitric oxide is an enzymatically produced free radical being synthesized by a variety of NOS with role in the regulation of vascular tone, neurotransmission, and immunity [4]. It is formed enzymatically from L-arginine (Figure 4).

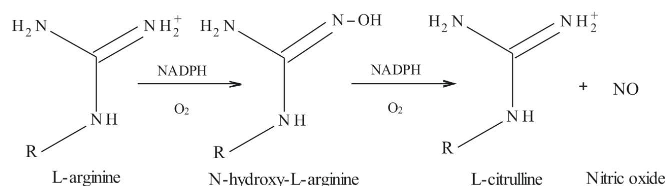


Fig. 4. Nitric oxide formation

eNOS and iNOS isoforms are two different variant expressed by adipocytes. In the lack of sufficient amounts of substrates they can also produce $O_2^{\bullet-}$ [6]. Aging can decreased eNOS availability and reduce NO synthesis causing endothelial dysfunction and impaired ventricular contractility in elderly patients [13].

Physiological effects of NO are the relaxation of smooth muscles in blood-vessel and macrophages activation having role in triggering the primary immune protection. An excess of NO is cytotoxic. In can react directly with biomolecules, or combine with $O_2^{\bullet-}$ to form peroxynitrite, able to induce lipid peroxidation, protein nitration and oxidation and DNA damage [2].

1.6. Carbonate radical ($CO_3^{\bullet-}$)

The formation of carbonate radical is homolysis of nitrosoperoxycarbonate, which is formed from peroxynitrite and carbon dioxide (Figure 5) [14].

Fig. 5. The formation of carbonate radical

$CO_3^{\bullet-}$ is more stable and less reactive than hydroxyl radical, it can diffuse from the site of origin and thus propagate the oxidative damage.

The reduction of $CO_3^{\bullet-}$-concentration is carried out by Mn-porphyrin (MnP), which protect mitochondria from peroxynitrite-mediated toxicity [4].

The carbonate radical can initialize the quick tyrosine oxidation or the oxidation of the sulfhydryl groups (Figure 6).

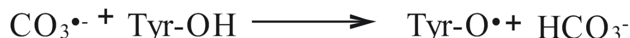


Fig. 6. Tyrosine oxidation

$CO_3^{\bullet-}$ can initialize the oxidative damage through the generation of free radicals through its reaction with nitrite (Figure 7).

Fig. 7. Nitrogen dioxide generation initiated by carbonate radical

NO_2 can initialize hydrogen abstraction from the fatty acids, tyrosine and tocopherol, initializing other free radical reactions [14].

1.7. Hydrogen peroxide (H_2O_2)

H_2O_2 is formed by the two-electron reduction of O_2. It is naturally produced in organism as a by-product of oxidative metabolism. In the presence of O_2 and transition metal ions, it can generate $HO^\bullet$ radical through the Fenton reaction. It is converted to water by the catalase enzyme. Recent studies have demonstrated that H_2O_2 influences the expression of genes through the apoprotein-1 (APO-1) and nuclear factor κB (NFκB) pathways [2].

1.8. Peroxynitrite ($ONOO^-$)

Peroxynitrite is a radical-radical combination reaction product form by linkage of nitric oxide (NO) with superoxide ($O_2^{\bullet-}$). It is a non-radical reactive species, however is more harmful than its parent molecules (Figure 8) [5].

Fig. 8. Peroxynitrite generation reaction

The reaction between $O_2^{\bullet-}$ and NO takes place spontaneously even in the presence of SOD. The reaction rate constant of peroxynitrite formation ($k_1 \sim 10^{10}\ M^{-1}.s^{-1}$) is with about one order of magnitude upper than the hydrogen peroxide and oxygen generation ($k_2 \sim 10^9 M^{-1}.s^{-1}$). Membrane permeability of $ONOO^-$ is highly pH dependent. At the normal blood pH (pH~7,4) 80% of peroxynitrite is in

the ionized form but at the pH~6,2 (inside a macrophage phagocytic vacuole) approximately 80% is in ONOOH form. ONOOH is a strong oxidant and react with nucleophile groups (thiols, iron/sulfur centers) but also react directly with molecules with a partial positive charge [5]. The relatively labile O-O bond in the ONOOH structure allows the formation a two strongly oxidizing ($HO^•$) and nitrating (NO_2) species.

Another way for the formation of free radicals from peroxynitrite is the reaction with carbon dioxide (CO_2) to produce nitrosoperoxycarbonate anion ($ONOOCO_2^-$), which rapidly homolysis into the $CO_3^{-•}$ and NO_2. This reaction occurs most frequently within mitochondria, where during the decarboxylation reactions a greater amount of CO_2 is synthesized.

The reaction of peroxynitrite with transition metal center is the third type of reaction of $ONOO^-$ in biological systems. These elements can be part of metalloproteins or metal complexes [4]. These reactions yield NO_2 and a strongly oxidizing oxo-metal complex [5].

The most important effects of peroxynitrite and peroxynitrite-derived radicals are the reactions with biological molecules such as lipids, proteins and nucleic acids. $ONOO^-$ reactions with mitochondrial components directly influences the activity of electron transport chain complexes and ATPase thus changing mitochondrial calcium homeostasis and contributes to $O_2^{•-}$ formation.

Peroxynitrite-mediated oxidation of mitochondrial membranes involves the release of pro-apoptotic factors. The cytotoxicity is mediated by a multitudinous of effects such as protein nitration and oxidation, lipid peroxidation, DNA oxidation, activation of matrix proteinase, inactivation of a series of enzymes [5]. Tyrosine nitration is considered a primary cause of peroxynitrite-mediated cytotoxicity. $ONOO^-$ does not react directly with tyrosine. The secondary radicals arising from peroxynitrite ($HO^•$, $CO3^{-•}$, NO_2, lipid peroxyl radicals) are responsible for protein tyrosine nitration and oxidation. The nitration of tyrosine has two steps. First a hydrogen atom is abstracted from tyrosine forming a tyrosyl radical. After that, the formed radical combines with NO_2, to produce 3-nitrotyrosine.

The most important negative effect of tyrosine nitration is the protein structure and function modification: changes in the catalytic activity of enzymes, altered cytoskeletal organization and cell signal transduction [5]. 3-nitrotyrosine levels can be used as an indicator of the reactive nitrogen species quantities in vivo. The second cause of peroxynitrite-mediated cytotoxicity can be the lipid peroxidation in membranes, lipoproteins and liposomes by abstraction a hydrogen atom from polyunsaturated fatty acids. Through formation of various nitrated lipids and intermediate products such as 4-hydroxynonenal and isoprostanes, a secondary oxidative insult may occur [5]. Being a strong oxidant, peroxynitrite can directly oxidize sulfhydryl groups to disulfides. Through these reactions many enzymes are inactivated [14].

Besides these adverse effects, the peroxynitrite-mediated cytotoxicity may be used by cells of the immune system. $ONOO^-$ and peroxynitrite-derived radicals can nitrate and/or oxidize proteins from different infecting microorganisms (ex. Escherichia coli, Trypanosoma cruzi etc.), the diffusion of peroxynitrite from the macrophage to the pathogen being highlighted in several scientific research [4].

1.9. Hypochlorous acid (HOCl)

HOCl is generated mainly by the MPO. It is a hem-containing enzyme that produces HOCl from H_2O_2 and chloride and chlorinated biomolecules. In human atherosclerotic lesions both MPO and HOCl modified proteins, respectively HOCl modified lipids are present. The HOCl present in biological systems can destroy the membranes of bacteria having an important role in the immunity.

2. Oxidative stress biomarkers

The oxidative stress markers are used to test oxidative injury in biomolecules and are the primary and secondary products of free radical damage [11].

2.1. Biomarkers of lipid peroxidation

The lipid peroxidation is the most extensively investigated process in tissue injury induced by free radicals. The most prevalent methods for measuring the lipid peroxidation level, are the methods that measure the levels of secondary oxidation products, such as: malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 2-propenal (acrolein) and isoprostanes [11].

Aldehydes can be absorbed from the diet, so the quantitative determination of these components should not be used as a biomarker unless diet is strictly controlled [15].

2.1.1. Malondialdehyde level

MDA is a preferential biomarker used to quantify the damage caused by free radicals to cellular lipids. MDA is the most toxic and mutagenic from the aldehydes generated by lipid peroxidation [16]. It reacts with deoxyadenosine and deoxyguanosine in DNA, forming DNA adducts (the primary one being M1G) [2]. The structural modifications of proteins by MDA have highlighted clinical relevance in atherosclerosis.

MDA can be measured from different biological samples (plasma, urine, tissue) by a direct or indirect HPLC method [11]. The quantitative determination of MDA is based on the reaction between aldehydes and thiobarbituric acid (TBA) when a red condensation product which shows strong absorption at 522 nm is formed [2]. MDA can be detected also with a direct method, but the signal given by MDA is much less that $MDA\text{-}(TBA)_2$ signal [15].

2.1.2. 4-OH-nonenal level (HNE)

HNE is an indirect index of oxidative damage, which can be measured in plasma and urine. It is generated by free

radical attack on omega-6 polyunsaturated fatty acids (arachidonic acid, linoleic acid and linolenic acid). HNE is permanently formed at physiologic conditions, but it is produced in larger quantities in various pathological conditions. Protein carbonyl can result from covalent attachment of HNS to proteins [7].

In the neurodegenerative diseases, atherosclerotic lesions and inflammatory diseases the concentration of both free and protein-bound HNE are 3- to 10-fold higher than the physiologic amount [15].

4-HNE can be measured from the human serum, after a fluorescent labeling of with 4-(N,N-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole, or with 4-(2-carbazoylpyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole (NBD-ProCZ). [17, 18].

2.1.3. 2-propenal (acrolein)

Acrolein is present in various environmental sources, the largest source being cigarette smoke. It forms stable covalent adduct with histamine, lysine and cysteine. Acrolein levels are increased in cardiovascular disease. It reacts with lysine of apolipoprotein A-I, and the carbonylation of modified apoA-I play a critical role in atherogenesis [15].

Acrolein can be detected from biological samples (urine, plasma, tissue) by a direct GC-MS method, or by an indirect HPLC-UV, HPLC-FD method after a preliminary reaction with different derivatizing reagents such as: m-aminophenol, hydroxylamine, Luminarin 3[a] or 2,4-dinitrophenylhydrazine [19,20,21,22].

2.1.4. Isoprostanes (IsoPs)

Isoprostanes are a family of prostaglandin like compounds generated in vivo by free radical catalyzed peroxidation of esterified arachidonic acid. Quantification of F2-IsoPs in either plasma or urine gives a highly precise and accurate index of oxidative stress. The quantitative determination is technically complicated. F2-IsoPs are stable but they are rapidly metabolized and eliminated. Several methods (GC-MS, LC-MS, LC-MS/MS, enzyme immunoassays) have been tried in the determination of isoprostanes. Although the MS methods are considered as "the gold standard" for the quantification of F2-IsoPs, the most used methods are the enzyme immunoassays due to their low cost and relative ease of use [15].

2.2. Biomarkers of protein oxidation

Proteins are major targets for reactive oxygen species because they can scavenge the majority (50%-75%) of them [15].

2.2.1. Carbonylated proteins level

The most likely amino acid residues to form carbonyl derivatives are lysine, arginine, proline, and histidine. Carbonylated proteins have a high chemical stability (stabile for 10 years at -80 °C), so they are very useful as markers of oxidative damage [15].

The classic method for the determination of protein carbonyl groups is the spectrophotometric quantification at 370 nm of hydrazones, formed by the reaction between carbonylated proteins and 2,4- dinitrophenylhydrazone (DNPH) [23].

Protein-DNPH can be detected also by immunochemical techniques using a polyclonal anti-DNPH antibody [24]. The ELISA method for protein carbonyls is more sensitive and discriminatory than the colorimetric assay [25].

2.2.2. Biomarkers of oxidative stress derived from tyrosine

Tyrosine is the most affected amino acid giving rise to different residues (through oxidation, nitration or halogenation) which may be used as biomarkers of protein damage. The most common modified proteins are: 3-nitrotyrosine (NO_2-Tyr) a stable marker of NO derived oxidants, 3-chlorotyrosine (Cl-Tyr), 3-bromotyrosine (Br-Tyr) and dityrosine (di-Tyr).

For the determination of NO_2-Tyr are used semi quantitative immunologic methods, that have a low specificity. Another method for its determination is the HPLC-UV, but is inadequate sensitivity and specificity for biological materials. The widely used methods for NO_2-Tyr determination are: HPLC with electrochemical detection, LC-MS/MS, electron capture–negative chemical ionization (EC-NCI) GC-MS, and GC-MS/MS.

Also LC-MS/MS and EC-NCI GC-MS methods are used for Cl-Tyr and Br-Tyr determination. EC-NCI GC-MS is much more sensitive as other methods, and allows simultaneous determination of these biomarkers.

For the di-Tyr determination several analytical methods were tested: HPLC-UV, HPLC-fluorescence, GC-MS after derivatization, LC-MS/MS with atmospheric pressure chemical ionization (APCI). The most appropriate method for the determination of di-Tyr from the biological samples is the triple-quadrupol LC-APCI-MS/MS which does not require any pretreatment other than centrifugation of the urine sample and addition of an internal standard [15, 26].

2.3. Biomarkers of oxidative damage of DNA

The most frequently product of hydroxylation of purine and pyrimidine bases from DNA are 8-hydroxy-2' -deoxyguanosine (8-OHdG), 8-oxo-7,8-dihydro-2' -deoxyguanosine (8-oxodG) and its free base 8-hydroxyguanine [15]. These product are formed by one electron abstraction by $OH^{\bullet}$ from the nucleobases of the DNA. The 8-OhdG quantitative determination can be done with high sensitivity by HPLC, GC-MS, LC-MS/MS, immunohistochemical methods (ELISA) and single cell gel electrophoresis. It can be determined from animal or human samples, such as urine, leukocyte DNA, plasma [27].

Two other products of oxidative damage to DNA are thymine glycol and thymidine glycol. These products may become biomarkers of DNA deterioration through oxidative stress. These products are eliminated from blood into urine without further transformation [15].

2.4. Other biomarkers

2.4.1. Myeloperoxidase (MPO) activity determination

MPO is a reactive oxygen species generating enzyme, which is stored within the neutrophils and monocytes. H_2O_2 serves as substrate for MPO in the generation of microbicial hypohalous acids. Hypohalous acids can generate the carbonyl groups on proteins. The carbonylation of neutrophil proteins is dependent on myeloperoxidase activity.

Several recent studies have demonstrated the possibility to use this enzyme as an oxidative stress marker. Recent clinical studies have shown that a MPO blood level increase leads to unbeneficial effects as cardiovascular damage. So the MPO serum level is a predictor of risk in patients with acute coronary syndromes, and is considered a marker of plaque vulnerability [3, 28].

2.4.2. Glutathione (GSH) level

GSH (L-γ–glutamyl-L-cysteinyl-glycine) is intracellular synthetized and its degradation occurs in the extracellular space. It is a low molecular weight thiol, which is readily oxidized to glutathione disulfide (GSSG) by ROS or RNS. The GSH level is reduced in oxidative stress, so its concentration can be used as a biomarker of oxidative injury. GSH/GSSG ratio is the most important redox couple that determines the antioxidant capacity of cells and it is used as an indicator of the cellular redox state [29, 30].

Estimation of the total amount of glutathione (GSH+GSSG) can be determined by the Ellman's method. In the first step the supernatant is reduced with the potassium borohydride to prevent the autoxidation of GSH. After this GSH is coupled with 5,5'-dithiobis-2-nitrobenzoic acid in sodium citrate to formed 5-mercapto-2-nitrobenzoic acid, and the modification of absorbance is read at 412 nm (Figure 9). The ration of reaction is proportional to the GSH and GSSG amount [31, 32, 34].

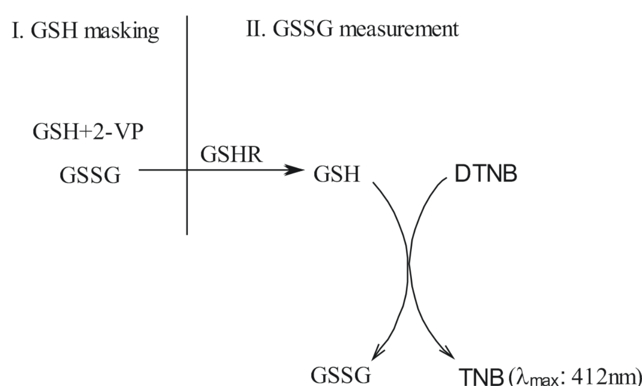


Fig. 9. GSH and GSSG determination

By using 2-vinylpyridine or its derivative (l-methyl-2-vinylpyridinium trifluoromethanesulfonate) as masking reagent, to block any free GSH in the sample, oxidizing GSH (GSSG) can be determined with the same method, described above. GSH can be quantitatively determined by subtracting GSSG from the total amount of glutathione. The reaction buffer also contains NADPH+H, glutathione reductase for GSSG reduction and GSH standard solution.

Other methods used glyoxalase I as reduced glutathione formation reagent which can be monitored directly at 240 nm.

The third assay involves o-phthaldialdehyde, which forms a fluorescent complex with GSH and can be monitored at 350 nm–420 nm [35, 33].

2.4.3. Metallothioneins (MT) as biomarkers of oxidative stress

MT is cysteine rich proteins, which are induced in organism succeeding metal exposure. It has important roles in the detoxification mechanisms of organism, through free radical scavenging activity and metal binding ability.

3. Defense mechanisms against free radicals

It is generally believed that diseases caused by oxidative stress should be treated with antioxidants [10]. All human cells protect themselves against free radical damage by enzymes (ex. superoxide dismutase, catalase, etc.), or spin traps (ascorbic acid, tocopherol, glutathione, etc.). The most important defense lines against free radicals are: SOD, CAT, GPx, glutathione reductase, glutathione transferase, thiol-disulfide oxidoreductase and peroxiredoxina.

3.1. Superoxide dismutase (SOD)

SOD is a metalloproteinase enzyme, involved in the defense system against free radical damage. Depending on the metal ion cofactor, it is present in organism under three isoforms:

- CuZnSOD (SOD1) → it is a protein present in endoplasmic reticulum and cytosol, and neutralize the superoxide anion locally synthesized.
- MnSOD (SOD2) → the mitochondrial protein neutralize the superoxide anion synthesized during the mitochondrial oxidative phosphorylation.
- ECSOD (SOD3) → it is an extracellular enzyme, found in the interstitial spaces of tissues and also in extracellular fluids, it is responsible for the majority of the SOD activity in plasma, lymph and synovial fluid [8].

SOD present at different levels transform superoxide anion radical to oxygen and H_2O_2, which is later reduced to H_2O by catalase, glutathione peroxidases or peroxiredoxina [3, 36, 37]. This reaction is the primary antioxidant defense line because it prevents further generation of free radicals [8].

If H_2O_2 is present in a high amount due to increased synthesis or because it is not decomposed by other enzymes, it can damage SOD. In the presence of bicarbonate the intermediated form of SOD, with free radical character is converted to unmodified enzyme and carbonate radical (Figure 10).

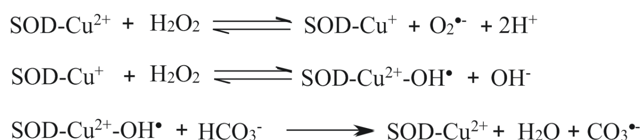


Fig. 10. SOD damage by H2O2

While SOD is protected in the present of HCO_3^-, the generated $CO_3^{\bullet-}$ can initiate the oxidative damage [14]. The SOD-Cu^{2+}-$OH^{\bullet}$ is similarly reactive as a hydroxyl radical and can inactivate other molecules of SOD by interaction with a histidine residue

3.2. Catalase (CAT)

CAT (hem protein) is an intracellular antioxidant enzyme, responsible for detoxifying hydrogen peroxide [38]. It is located in the peroxisome and cytosol. It is activated especially when the glutathione content is limited or the GPx activity is reduced. CAT catalyzes the reaction in which H_2O_2 form water and molecular oxygen [3].

3.3. Glutathione peroxidase (GPx), glutathione

GPx (a selenium-containing enzyme) is found in the majority in the erythrocytes, thrombocytes and leucocytes. It has importance in reducing the oxidative stress through reduction of hydrogen peroxide and lipid peroxides to water and lipid alcohols; at the same time it oxidizes glutathione to glutathione disulfide [3]. The most important substrate of GPx is the glutathione. GPx among other effects catalyzes the GSH-dependent reduction of H_2O_2 and other peroxides [29].Together they have importance in the detoxification of free radicals such as hydroxyl radical, lipid peroxyl radical, peroxynitrite radical and H_2O_2.

GSH is synthesized in the cytosol from glutamate, cysteine and glycine, catalyzed by gamma-glutamylcysteine synthetase (GCS) and GSH synthase. After its synthesis, a certain amount is delivered into the mitochondria and endoplasmic reticulum and the rest reaches the blood plasma, exocrine secretions, lung lining fluid and cerebrospinal fluid [39].

The GSH functions as an antioxidant in the organism. It can scavenge free radical species in circulation, but also serves to facilitate the recycling of oxidized α-tocopherol and ascorbic acid. GSH is often used as a biomarker of circulating antioxidant levels [16].

Glutathione is involved in several cellular reactions:

- GSH scavenge reactive oxygen species ($LOO^{\bullet}$, $ONOO^-$, H_2O_2, $HO^{\bullet}$, etc.) through it oxidation to GSSG, which is then reduced to GSH by the NADPH-dependent glutathione reductase (Figure 11).
- GSH form mercapturates by it reaction with physiological metabolites and xenobiotic. These reactions are initiated by GSH-S-transferase.
- GSH protects NO from oxidative decomposition
- GSH serves as a substrate for formaldehyde dehydrogenase releasing S-formyl-GSH
- GSH is important also for the immune response activating the T-lymphocytes [29].

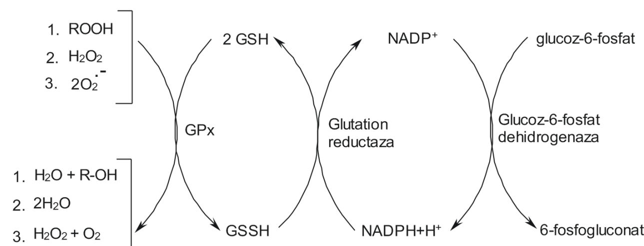


Fig. 11. GSH function in the organism

3.4. Uric acid

The uric acid appears in organism through the metabolic breakdown of purine nucleotides. It is an endogenous substrate of myeloperoxidase. Uric acid has a strong protector effect against peroxynitrite-mediated damage. The reaction between uric acid and $ONOO^-$ is relatively slow; its effect comes from scavenging the peroxynitrite-derived radicals and preventing of Tyr nitration [4].

4. Therapeutic efforts to reduce oxidative stress

4.1. Vitamins with antioxidant properties (vitamin C, E and carotenoids)

Vitamin C is considered the most important water-soluble antioxidant which is present in the cells and in the extracellular fluids; the human plasma contains ~ 60 μmol ascorbate. Ascorbic acid is a strong reducing agent. It is capable to transfer two electrons in steps to electrophilic radicals. It can be considered as an enol where the deprotonated form is a stabilized enolate. It is capable to scavenge hydroxyl radical, superoxide radical, hydrogen peroxide and singlet oxygen. By the interaction with ROS dehydro-ascorbate is formed through the ascorbyl free radical formation. After that it is reduced back to ascorbic acid [2, 40, 41].

Vitamin E is the major lipid-soluble antioxidant which protects membrane fatty acids from lipid peroxidation [40]. Tocopherol group have eight isomers. The best known isomer is, α-tocopherol, is present in the membrane of cells and cell organelle. It is capable to donate the hydrogen from the hydroxyl group to the lipid peroxyl radical. The free radical formed from vitamin E is stabilized through delocalization of the solitary electron over the aromatic ring structure [2]. Vitamin C is capable of regenerate vitamin E from tocopheroxyl radical [41, 42].

Carotenoids and its derivatives (ß-carotene, lycopene, lutein, b-cryptoxanthin, zeaxanthin, astaxanthin) are natural colorants synthesized by plants and have antioxidant effect especial in lipid-rich tissues. They are not particularly good scavengers of peroxyl radicals, but are exceptional in trapping singlet oxygen and other reactive species at which most other antioxidants are relatively useless. The antioxidant properties are given by the many conjugated carbon

double bonds. It has synergistically effects with vitamin E [2, 40].

4.2. Polyphenols, flavonoids, resveratrol

Polyphenols are the main antioxidant constituents of fruits, vegetables, cereals, olive, chocolate, tea, coffee and wine. They can be grouped by their structures into phenolic acids, flavonoids, stilbenes and lignans. A common physiological effect is the anti-atherosclerotic property in the early stages of platelet aggregation. Many polyphenols have direct antioxidant effect, reacting with reactive oxygen species and forming products with lower reactivity. It can also influence indirectly the redox status by activating the endogenous antioxidant status or by inhibiting ROS generating enzymatic systems.

Flavonoids have polyphenol structure. They have beneficial effects for the prevention and treatment of atherosclerosis and CVDs, by having in their structure phenolic hydroxyl groups, which act as electron donor, and who are responsible for free radical scavenging activity. Flavonoids are part of human diet. Their daily total intake (~1g) is with one order of magnitude higher than all other classes of phytochemicals (vitamin C, vitamin E and beta-carotene intake from food is estimated to 100mg). Flavonoids can be grouped into: flavones, flavanols, flavonols, flavanones, anthocyanins and isoflavones. Quercetin and catechin are the most widely distributed flavonoids which are abundant in red wine, tea, onions and chocolate [43].

Resveratrol is another natural component in grapes and grape products with antioxidant effect. Treatment with resveratrol leads to: increase in the activities of superoxide dismutase, catalase, glutathione, glutathione reductase, glutathione peroxidase and glutathione-5-transferase [43]. Resveratrol is thought to have various antiantherogenic activities (inhibition of LDL oxidation, inhibition of platelet aggregation, regulation of vascular smooth muscle proliferation). It inhibits endothelial activation, monocyte adhesion and decrease in proinflammatory gene expression by inhibition of NF-κB activation in coronary arterial endothelial cells [13].

Xanthones, present in more plants have a heterocyclic compounds with the dibenzo-γ-pyrone structure. It has several –OH group, serving as H donors, and double bounds being able to form free radicals with increased stability. Xanthones are able to scavenge $O_2^{\bullet-}$, $HO^{\bullet}$ and $ONOO^-$. They have a lower IC_{50} for superoxide than the glutathione, but their free radical scavenging activity is shown in several studies [44].

4.3. Nitrone-based antioxidants (PBN, NXY-059, STAZN)

PBN (N-tert-Butyl-α-phenylnitrone) is a nitrone-based antioxidant that scavenges a wide variety of free radical species (especially $O_2^{\bullet-}$, $HO^{\bullet}$), increase endogenous antioxidant levels and inhibits free radical generation. Several studies have shown that the mediators of inflammatory ROS (MDA, HNE, myeloperoxidase activity, xanthine oxidase activity, Nox activity) are depressed in PBN-treated rats [24,45]. Jian-Jun Wen at al. has demonstrated a protective activity of PBN in the acute Chagasic myocardium and against mitochondrial dysfunction-associated ATP homeostasis. PBN was administrated 50 mg/kg by intraperitoneal injection [45].

Disodium 2,4-disulphophenyl-N-tert-butylnitrone (NXY-059) is also an nitrone-based antioxidant, which reduces infarct volume and was neuroprotective in experimental stroke models (rodents, rabbits, primates) but ineffective in a large clinical trial. The beneficial effects of this substance were demonstrated in several preclinical studies, but could not be demonstrated in clinical trials (SAINT2) [46,47,48].

Stilbazulenyl nitrone (STAZN) is a new nitrone antioxidant, which ensures functional and morphological neuroprotection in brain ischemia (0,7 mg/kg), and confers marked cardioprotection in acute coronary ischemia, when it is given immediately prior to reperfusion [49, 50].

4.4. Physical exercise

Several studies have demonstrated that physical activity prevents the progression of CVDs and common tumors. It slows the atherosclerosis progression, prevent plaque rupture. These positive effects are attributed to exercise increase in blood antioxidant capacity through high hydrophilic antioxidants (uric acid, bilirubin and vitamin C) level [43].

Conclusions

Large number of research papers show that oxidative stress can lead to pathological conditions as CVDs, diabetes, neurodegenerative diseases. Therapeutic strategies are limited to the use of natural occurring antioxidants. In some cases even synthetic substances able to capture free radicals are tried in human studies (brain ischemic processes). So far synthetic substances, as NXY-059, were tried only in acute conditions leaving the chronic oxidative stress without any treatment options.

Acknowledgement

This paper is supported by the Sectoral Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/159/1.5/S/133377/

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