Summary: The presence of free radicals in biological material has been discovered some 50 years ago. In physiological conditions, free radicals, in the first place the ones of oxygen and nitrogen, are continuously synthesized and involved in the regulation of a series of physiological processes. The excess of free radicals is efficiently eliminated from the body in order to prevent their toxic effects. Toxic effects of free radicals may be classified into three groups: a) change of intracellular redox potential, b) oxidative modification of lipids, proteins and DNA, and c) gene activation. Lipid peroxidation involving cell membranes, lipoproteins and other molecules leads to the production of primary high-reactive intermediaries (alkyl radicals, conjugated dienes, peroxy- and alkoxyl radicals and lipid hydroperoxide), whose further breakdown generates the secondary products of lipid peroxidation: short-chain evaporable hydrocarbons, aldehydes and final products of lipid peroxidation: isoprostanes, MDA, 4-hydroxy-2,3-transnonenal and 4,5-dihydroxydecenal which are important mediators of atherosclerosis, coronary disease, acute myocardial infarction, rheumatoid arthritis, systemic sclerosis and lupus erythematoses. Oxidative modification of proteins is manifested by changes in their primary, secondary and tertiary structures. Proteins have a specific biological function, and therefore their modification results in unique functional consequences. The nature of protein modification may provide valid information on the type of oxidants causing the damage. Chlorotyrosyl is a specific marker of oxidative damage to tyrosine caused by HOCl action, which most commonly reflects the involvement of neutrophils and monocytes in oxidative stress, while nitrotyrosyl indicates the presence of higher peroxy-nitrite synthesis. Methionin and cysteine are the amino acids most sensitive to oxidative stress, carbonyl groups are markers of severe damage caused by free radicals, and di-tyrosyl is the most significant and sensitive marker of oxidative modification made by γ-rays. "Carbo-

Kratak sadržaj: Prisustvo slobodnih radikala u biološkim materijalima je otkriveno pre nešto više od 50 godina. U fiziološkim uslovima, slobodni radikali, pre svega kiseonik i azot, stalno se sintetizuju i pri tom učestvuju u regulaciji niza fizioloških procesa. Višak slobodnih radikala se efikasno uklanja iz organisma u cilju sprečavanja njihovih tocišćnih efekata. Toksичni efekti delovanja slobodnih radikala se mogu podeliti u tri grupe: a) pomeranje intračelijinskog redoks stanja, b) oksidativna modifikacija lipida, proteina i DNK i c) aktivacija gena. Lipidna peroksidacija koja zahvata čelijске membrane, lipoproteine i druge molekule dovodi do stvaranja primarnih visokoreaktivnih intermedijera (alkil radikala, konjugovanih diena, peroksi i alkoksi radikala i lipidnih hidroperoksid), koji daljom razgradnjom daju sekundarne produkte lipidne peroksidacije: kratkolančane isparljive ugljovodonike, aldehide i krajnje proizvode lipidne peroksidacije: isoprostane, MDA (malonaldehid), 4-hidroksi-2,3-transnonenal i 4,5-dihidroksidecenal, koji su značajni medijatori ateroskleroze, koronarne bolesti, akutnih infarkta miokarda, reumatoidnog artrita, sistemskih skleroze i lupusa eritematodesa. Oksidativna modifikacija proteina manifestuje se promenama njihove primarne, sekundarne i terciarne strukture. Proteini imaju specifičnu biološku funkciju, stoga njihova izmena ima jedinstvene funkcionalne posledice. Pritroda proteinskih modifikacija može nam dati validan podatak o vrsti oksidansa koji je doveo do oštećenja. Hlorotirozil je specifičan marker oksidativnog oštećenja tirozina dejstvom hipohlorita (HOCl), koji najčešće reflektuje učešće neutrofila i monocita u oksidativnom stresu, dok nitrotnirozil ukazuje na prisustvo povećane sinteze peroksinitrita. Metionin i cistein su najzastupljeniji aminokiseline na dejstvo oksidativnog stresa, karbonilne grupe su markeri teškog oštećenja slobodnim radikalima, dok je di-tirozil najznačajniji i najzastupljeniji marker oksidativne modifikacije dejstvom γ-zraka. "Karbonilni stress" je značajan vid sekundarne oksidacije proteina u kojem redukujući šećeri neenziemski reaguju s aminogrupama proteina i lipida dovodeći do stvaranja kovalentnih jedinjenja poznatih kao uzapredovani proizvodi glikozilacije ili AGE-proizvodi (advanced glycosylated end products). Hidroksilni radikal oštećuje i molekul DNK dovodeći do gubitka baze i stvaranja abazičnih mesta (AP site-ova), ceapa lanca DNK i modifikacije šećera. Krajnji proizvodi lipidne peroksidacije (MDA) mogu se kovalentno vezivati za DNK, stvara-
**Introduction**

The presence of free radicals in biological materials was discovered some 50 years ago (1). Soon after that discovery, Harman (2) reported his hypothesis that free radicals were the products of enzymatic reactions in vivo, describing them as a «Pandora's box», responsible for the majority of cell damages, mutagenesis, carcinogenesis and degenerative processes inducing the aging (3).

The research of free radicals began its second era when McCord and Fridovich (4), in 1969, discovered the enzyme superoxide dismutase (SOD), and documented the significance of free radicals for biological systems. Many studies then initiated were aimed at investigating the oxidative damage to DNA, proteins, lipids and other cell components caused by free radicals (5).

The third era of research on free radicals began with the first description of their biological effects. Mittal and Murard (6), in 1977, confirmed that superoxide anion (O$_2^-$), via its hydroxyl radical derivatives, stimulated the activation of guanylate cyclase and synthesis of the secondary messenger of cGMP. In 1987, Ignaro and Kadowitz (7) and Moncada (8) discovered an independent role of nitrogen monoxide (NO) as a regulatory molecule in the control of the relaxation of smooth muscle cells and inhibition of platelet adhesion. Roth and Dröge (9) proved that superoxide anion or a low concentration of hydrogen peroxide in activated T-lymphocytes increased the synthesis of interleukin-2 (IL-2), an important immunological protein of T-lymphocytes. Keyse and Tyrrel (10) found that hydrogen peroxide induced the expression of hem oxygenase gene (HO-1 gene). Storz and associates (11), in 1990, showed that hydrogen peroxide induced the expression of different genes in bacteria, while Schreck and Baeuerle (12), in 1991, described the activation of transcriptional nuclear factor -κB (NF-κB) in mammalian cells by H$_2$O$_2$ action.

Signal transduction is a process of transmission of signals from the cell surface into the cell interior. Free radicals and their metabolites are included in a complex network of different signal pathways and most probably operate as intracellular and intercellular mediators, transforming the initial signal into a biochemical response. Free radicals and redox stress also participate actively in cell signalization as secondary messengers in the activation of transcription factors and induction of gene expression.

The term «redox-signalization» is used in the description of a regulatory process where a signal originates during redox-reaction. The principle mechanism of redox homeostasis is based on the induction of redox sensitive signaling cascade which leads to a higher expression of antioxidative enzymes or to increased cysteine transport system, which results in an elevated intracellular glutathione concentration.

Cells and tissues are stable if the rate of production of free radicals and capacity of «cleansing» are in continuous balance. Redox signals demonstrate that such balance is impaired either by an increased production of free radicals or lower activity of one or more antioxidant systems. If the initial increase of free radicals were relatively small, the antioxidant response would be sufficient to neutralize it and restore the balance. In some circumstances, production of free radicals may be more intensive and persistent, therefore, the antioxidant response will not be adequate to neutralize them and restore the system to the initial level of redox-homeostasis. In such cases, the system may still be recovered and restore its balance, but the newly developed «quasi-stable» state is now followed by a high production of free radicals and varying gene expression. Pathological conditions...
occur exclusively in extreme situations, with continuously high concentrations of free radicals. These conditions do not necessarily mean an immediate loss of homeostasis, but rather a chronic shift of the homeostasis level. Pathological symptoms may result from the noxious effect of free radicals, as well as altered gene expression due to their effect (13–15).

Under physiological conditions, free radicals, in the first place oxygen and nitrogen, are constantly synthesized and simultaneously involved in the regulation of a series of physiological processes. An excess of free radicals is efficiently eliminated from body in order to prevent their toxic effects. The toxic effects of free radicals are associated with the development of over 100 different diseases such as: cardiovascular diseases, myocardial infarction, atherosclerosis, diabetes, neurodegenerative diseases, rheumatoid arthritis, cystic fibrosis, etc. (16). The toxic effects of free radicals may be divided into three groups:

1. shift of intracellular redox condition
2. oxidative modification of lipids, proteins and DNA
3. gene activation.

**Lipid peroxidation**

Lipid peroxidation is an oxidative damage involving the cell membranes, lipoproteins and other molecules containing lipids under conditions of oxidative stress. Cell membrane lipids (phospholipids, glycolipids and cholesterol) are the most common substrates of oxidative attack. Once initiated, the reaction of peroxidation is continued autocatalytically, and has a prodigious course, finally resulting in structural-functional changes of substrates (17).

Lipid peroxidation in controlled conditions modifies the cell membrane permeability, affects the intensity of the metabolism of membrane lipids and proteins, and provides control of the synthesis of biologically active eicosanoids, control of cell proliferation and initiation of cell death (18, 19).

Polyunsaturated fatty acids in phospholipids and glycolipids are the basic substrate of oxidative damage to lipids caused by free radicals. The process of lipid peroxidation proceeds through the phases of initiation, propagation and termination.

**Initiation** of the lipid peroxidation process implies an elimination of a hydrogen atom from a methyl group (–CH3), which is in an α-position in relation to the –C=C– bond in the chain of fatty acids (LH), in which process a lipid radical (L·) is produced. With the aim of molecule stabilization, double bonds are arranged intramolecularly in lipid radicals and conjugated dienes are formed. Addition of a molecular oxygen to conjugated dienes produces a peroxy radical (LOO·). A peroxy radical may further initiate the removal of hydrogen from adjacent lipid molecules, at which point the reaction of lipid peroxidation enters the second phase of propagation and autooxidation, resulting in the formation of lipid hydroperoxide (LOOH) and new lipid (alkyl) radicals (20).

Lipid hydroperoxide is the next potential source of high-reactive OH·. In the presence of Fe2+ in the classical Fenton’s reaction, breakdown of LOOH yields OH· and alkoxyl (oxyl) radical (LO·).

\[
\text{LOOH} + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{LO}^·.
\]

In the presence of Fe5+ LOOH are degraded to peroxy radical (LOO·).

\[
\text{LOOH} + \text{Fe}^{5+} \rightarrow \text{LOO}^- + \text{H} + \text{Fe}^{2+}.
\]

Alkoxyl and peroxy radicals are responsible for further initiation and propagation of lipid peroxidation. Lipid hydroperoxides may be produced by one-electron reduction of oxyl radical (LO·) in a Fe2-mediated reaction (21).

There are three options for the further breakdown of alkoxyl (oxyl) radical:

1. by elimination of hydrogen from adjacent molecules of unsaturated fatty acids, an oxyl radical (LO·) is transformed into the hydroperoxyl radical (LOOH);
2. in the process of β-oxidation, they are transformed into aldehydes and new alkyl radicals;
3. oxygenation and rearrangement of double bonds result in epoxy peroxide radicals (OLOO·).

Malondialdehyde (MDA) is the final product of lipid peroxidation, which may react further with protein amino groups, forming the intramolecular crossover bonds, or may bind two different proteins through intramolecular bonds thus changing their structural-functional characteristics (22).

Lipid peroxidation reduces the fluidity of biological membranes which leads to their higher permeability for mono- and bivalent ions and inactivation of membrane enzymes (23). Fragmentation of fatty acids chains to intermediaries such as aldehydes and short-chain evaporable hydrocarbons (i.e., pentane) gives rise to the loss of membrane integrity, while the rupture of lysosomal membranes leads to a release of hydrolytic enzymes, which further damages the cell (24).

**End products of lipid peroxidation**

Lipid peroxidation products may be divided into primary high-reactive intermediaries (alkyl radicals, conjugated dienes, peroxo and alkoxyl /oxyl/ radicals and lipid hydroperoxide) and, upon further degradation, secondary products, i.e. short-chain evaporable hydrocarbons, aldehydes and end products of lipid peroxidation: isoprostanes, MDA, 4-hydroxy-2,3-trans nonenal and 4,5-dihydroxydecenal.
Malondialdehyde (MDA) is physiological ketoaldehyde, secondary product of peroxidative breakdown of unsaturated fatty acids, particularly arachidonic acid (25). MDA in a higher concentration reacts with free amino groups of proteins (especially with lysine residue). It is the path of the generation of MDA-modified proteins, which have altered biological characteristics. Such modified protein structures have immunogenic features. It has been validated by the isolation of autoantibodies against MDA-modified lysine residue in human and animal serum (26). Some studies have shown that an increased titer of these autoantibodies directly correlates with the extent of damage and may predict the progression of atherosclerosis, coronary disease and acute myocardial infarction. Reaction of MDA and proteins is especially important for atherosclerosis, which is the major cause of coronary heart disease and brain insult. Bonding of MDA to LDL particle initiates several proinflammatory and proatherogenic processes, leading to the generation of foam cells (27). MDA concentration is elevated in diabetes mellitus, plasma and atherosclerotic plaque as well as the concentration of new compounds developed by bonding of MDA to the lysine residue of apolipoproteins B-100 and 4,5-dihydroxy nonenal (HNE) (28, 29). Higher MDA concentrations were found in pre-eclamptic women (30), in plasma samples and expired air of asthmatics (31), in patients with Parkinson’s disease, amyotrophic lateral sclerosis and Alzheimer’s disease (32).

4-hydroxyynonenal (HNE) is a major and most toxic aldehyde, which is produced by the oxidation of α-6 polynsaturated fatty acids (arachidonic, linolic and linolenic acids) (33), and considered the second toxic messenger of free oxygen radicals (34,35). HNE reacts with proteins, peptides, phospholipids and nucleic acids, and, therefore, it has multiple cytoxic, mutagenic, genotoxic and signal effects including the inhibition of proteins and DNA synthesis, enzyme inactivation, stimulation of phospholipase C and chemotaxis of neutrophils, modulation of platelet aggregation, as well as modulation of various gene expression (36). HNE is considered a significant mediator of apoptosis induced by oxidative stress, cell proliferation and signaling pathways (37). Physiologically, HNE is continuously synthesized in minimal quantities, but its synthesis is considerably enhanced in the pathological conditions of increased lipid peroxidation. In physiological conditions, HNE concentration ranges from 0.1 to 3.0 μmol/L. During intensive oxidative stress, such as rheumatoid arthritis, systemic sclerosis, lupus erythematoses, chronic lymphedema, chronic renal failure, the concentration of HNE rises 3–10 times above physiological concentrations (38, 39). HNE forms stable compounds with residues of histidine, lysine and cysteine (covalent bonds), known as advanced lipoxidation end products (ALE), and initiates the generation of protein carbonyl groups (40–42).

Acrolein is a α,β-unsaturated aldehyde which emerges as a secondary product of lipid peroxidation, but also in the reaction of oxidation of amino acids catalyzed by myeloperoxidases. It is present in a variety of human surrounding resources, particularly in cigarette smoke. Acrolein reacts with lysine residues of apolipoprotein A-I, the key protein of HDL particle, that has a critical role in mobilizing the cholesterol from macrophages localized on the blood vessel walls. Accordingly, the products of oxidative modification of proteins by acroleins are localized in human atherosclerotic lesions and have a significant role in atherogenesis (43). Past studies showed that exposure of vascular smooth muscle cells to different acrolein doses led to significant damage of cell morphology whose extent was relative to acrolein concentration and length of exposure (44). Higher acrolein concentrations (5–10 μg/mL) cause activation of MAP and tyrosine kinase, that has an important role in vascular pathogenesis and acrolein-induced cell damage. Acrolein inhibits the synthesis of interleukin IL-2, IL-10, granulocytic-macrophage colony stimulating growth factor, interferon γ, TNF-α and NF-kB1. The latter blocks bonding of DNA to NF-kB1, that has a direct implication to the modulation of gene expression and modification of gene transcription (45, 46).

Hexanoyl-lysine (HEL) is a recently discovered product of lipid peroxidation which is formed via an oxidative modification of the lysine residue of a protein by hexanoyl action. Hexanoyl lysine is generated by metabolizing of α-6 fatty acids (linolic and arachidonic) and it is a marker of the initial phase of lipid peroxidation. Numerous studies have verified the presence of hexanoyl lysine in oxidatively modified LDL particles, atherosclerotic lesions, and in human urine and serum. It has been proved that HEL may arise in muscles during intensive exercise. Antioxidants (e.g., flavonoids) may inhibit its synthesis.

Isoprostanes are prostaglandin F$_2$-like substances (F$_2$-IsoPs) which are produced in vivo by non-enzymatic peroxidation of arachidonic acid via the action of free radicals without cyclooxygenase action (47, 48). Isoprostane synthesis, during auto-oxidation of polysaturated fatty acids, was first described in mid 1970s of the 20th century, but their in vivo synthesis was not explained until 1990 (49, 50). F$_2$-isoprostanes are a group of 64 isomers, structurally similar to prostaglandin F$_{2α}$ (PGF$_{2α}$). Peroxidation of arachidonic acid first results in arachidonyl-radicals, which by endo-cyclization, yield 4 bis-cyclic endoperoxide intermediaries, similar to prostaglandin H$_2$ (PGH$_2$). Upon reduction of these intermediaries, 4 series of isoprostanes (series 5, 12, 8 and 15), i.e. 4 F-rings of regioisomers are formed; each of them consists of 8 racemic diastereoisomers. According to the nomenclature system approved by the Committee of Eicosanoids Nomenclature, the names of these series come from the number of C-atoms in the chain.
the OH-group is bound to, and where the C-atom of carboxyl group is designated as C-1 (51). \( \text{F}_2 \)-isoprostanes always develop esterified, in situ, in the composition of phospholipids. Upon the action of phospholipase \( \text{A}_2 \), they are released from phospholipid composition and pass into all body fluids (52). Rearrangement of first developed endoperoxides results in isoprostanes with \( \text{E} \) or \( \text{D} \) ring or compounds with thromboxane ring (53). Reactive cyclopentanes \( \text{A}_2 \) and \( \text{J}_2 \) isoprostanes (54) as well as acyclic ketoaldehydes, termed isoketals, are the result of \( \text{E}_2 \) and \( \text{D}_2 \)-isoprostanes dehydration, the latter being highly reactive and unstable compounds (55). The main metabolite of isoprostane in humans, which is excreted via urine, is \( 2, 3 \)-dinitro-5,6-dihydro-15-\( \text{F}_2 \)-isoprostane (56). Oxidation of docosahexaenoic acid (C\( _{22} \), 6\( \text{E} \)6\( \text{E} \)), most prevalent polyunsaturated fatty acid in the central nervous system, produces isoprostanelike compounds called neuroprostanes that may be a unique marker of oxidative CNS damage (57).

**Determination of lipid peroxidation end products**

The spectrophotometry method based on the measurement of MDA concentration in reaction with thiobarbituric acid was previously used very often for the assessment of the intensity of the lipid peroxidation process in \( \text{in vivo} \) and \( \text{in vitro} \) conditions (17, 20). The knowledge that MDA may bind to protein molecules and modify them, where newly developed complexes assume immunogenic features, has led to the development of tests which are based on immunological principles (28). MDA binding to DNA chain, particularly to guanine, adenine and cytosine bases, causes the formation of oxidatively modified DNA chains that may be determined by gas chromatography with mass detection (58, 59).

Determination of protein conjugates developed from a reaction with 4-hydroxynonenal and histidine and cysteine, respectively, is completed by means of liquid chromatography with electro-spray ionization together with mass detection or HPLC technique (60, 61), and recently ELISA has been used (62).

Acrolein bound to carnosine, homocarnosine or histidine, and especially to DNA chain, may be determined by liquid chromatography with mass detection (63) or liquid chromatography with electrospray ionization together with mass detection (64). Binding of acrolein to lysine residue of protein conditioned the development of ELISA assays using the monoclonal antibodies which specifically react with Acr-lys product, in the form of a competitive reaction with formaldehyde-hydro piperidine-lysine (FDP-Lys). Similar to acrolein-lysine, hexanoyl-lysine may be determined by an ELISA assay using the monoclonal antibodies which enable determination of this compound in serum, urine and even in tissues.

\( \text{F}_2 \)-isoprostanes are presently considered very reliable parameters of oxidative stress and lipid peroxidation \( \text{in vivo} \). Other products of the isoprostane pathway, such as \( \text{D}_2 \)- and \( \text{E}_2 \)-, are less stable, and consequently less used for determination. \( \text{F}_2 \)-isoprostanes may be determined in esterified form in all tissues, and in free form in all biological fluids, as a marker of the physiological level of oxidative stress (65). In order to examine the endogenous production of \( \text{F}_2 \)-isoprostanes in humans, it is necessary to determine free non-metabolized \( \text{F}_2 \)-isoprostane in biological fluids such as plasma and urine, esterified \( \text{F}_2 \)-isoprostane in tissue and plasma lipoproteins, as well as to measure the major metabolite \( \text{F}_2 \)-isoprostane in urine. The referent analytical procedure for the determination of isoprostanes in tissues and biological fluids is gas chromatography with mass detection (GC/MS), which is long-lasting and expensive. Alternative methods that have been developed over time are based on immunochemical and radioimmunochemical principles (66, 67).

Isoprostane determination has several advantages over the determination of other markers of oxidative stress. \( \text{F}_2 \)-isoprostanes are chemically stable, specific products of lipid peroxidation, produced \( \text{in vivo} \), their concentration is detectable in all tissues and biological fluids, therefore enabling the determination of a reference interval; their values significantly increase in oxidative stress, and their concentration is not dependable upon the volume of lipid intake (68, 69). Isoprostanes are commonly determined in urine, because the method is completely non-invasive, they are not subject to auto-oxidation and are very stable in urine (70). No significant daily variations of concentration or day-to-day variations have been reported in healthy people (17). \( \text{F}_2 \)-isoprostanes may be determined in the expired air condensate, what makes them very suitable for the determination of inflammatory conditions as well as the effect of oxidative stress on the development of pulmonary diseases in both children and adults with severe pulmonary function impairment (72, 73). Determination of esterified \( \text{F}_2 \)-isoprostanes in plasma lipoproteins may be a significant tool in the investigation of LDL particle oxidation \( \text{in vivo} \), as the central event of atherosclerosis (74).

Quantitative determination of \( \text{F}_2 \)-isoprostanes in tissues and different biological fluids (serum, plasma, urine, cerebrospinal and synovial fluids, bronchoalveolar lavage, etc.) is a new approach to the quantification of oxidative stress, which is the biological foundation for the application of adequate therapy in specific pathological conditions.

**Oxidative modification of proteins**

Proteins are highly sensitive to free-radicals action. The reactions of protein structure modification by the action of free radicals proceed in \( \text{in vivo} \)
conditions and are responsible for the physiological processes of aging, degradation and protein restoration, as well as the regulation of aerobic and anaerobic metabolism. Oxidative modification of proteins has been verified in the following diseases and conditions: aging, ischemic-reperfusion lesions, coronary and cerebral occlusion, pulmonary emphysema, cataract, rheumatoid arthritis, chronic alcoholism, neurological conditions followed by demyelination, diabetes mellitus, liver and kidney disorders, hemochromatosis, etc. (75–80). Unlike lipid peroxidation, modification of proteins is fast and linear (in time and concentration), and therefore it is a more sensitive parameter of oxidative modification of biomolecules than lipid peroxidation (81–83).

Oxidative modification of proteins is manifested by changes of their primary, secondary and tertiary structure. Modification of primary protein structure results from the modification or deficit of some amino acids, aggregation and fragmentation of proteins, while modification of secondary and tertiary structure causes the changes of solubility and charge (84, 85). Stadtman’s classification established several types of modification of proteins and enzymes, that are illustrated in Table I (Table I).

Oxidative modification of amino acids often leads to the development of some other forms of amino acids, what may affect the functional activity of the protein itself. Nevertheless, not every oxidative modification of amino acids is disastrous for its function. Oxidations regularly occur in the cell, which may lead to the production of amino acid derivatives that may be involved in further metabolic pathways in the body (Table II). Amino acids most susceptible to oxidative attack are cysteine and methionine: they both have the sulfur atom (86, 87). All kinds of oxidizing agents may induce modification of cysteine and methionine that is reflected in the formation of disulfide bonds, mixed disulfides and thiyl radicals. Other amino acids may also be oxidized but they require more strict oxidative conditions (88).

### Table I Types of oxidative modification of proteins (Stadtman’s classification).

| 1. loss of catalytic function |
| 2. modification of amino acids |
| 3. formation of carbonyl groups |
| 4. increase of acidity |
| 5. methionine conversion into methionine sulfide |
| 6. reduced thermostability |
| 7. changed viscosity |
| 8. fluorescence change |
| 9. fragmentation |
| 10. formation of interprotein bonds – aggregation |
| 11. formation of S-S bridges |
| 12. higher proteolytic sensitivity |

| Table II Oxidative modification of some amino acid residua. |
|-------------------|---------------------------------|
| Amino acid residue | Oxidatively modified amino acid residue |
| Arginyl           | Glutamyl semialdehyde            |
| Prolyl            | Glutamyl semialdehyde            |
|                   | Pyroglutamyl/Glutamyl            |
|                   | Cis/Trans-4-hydroxy prolyl       |
| Lysyl             | α-Amino adipyl-semialdehyde      |
| Histidyl          | Asparaginyl/Aspartyl             |
| Cysteinyl         | -S-S- Protein bonds              |
|                   | -S-S-R (thiyl-radicals)          |
| Methionyl         | Methionyl-sulphoxide             |
| Tyrosyl           | Tyrosyl-Tyrosyl interaction      |
| Leucyl            | Hydroxy-leucyl                   |
| Tryptophan        | N-formyl kynurenine              |
| Threonyl          | 2-amino-3-ketobutyric acid       |

### Direct oxidation of amino acids

Several systems have been evidenced in the cell which are involved in the oxidative modification of proteins. Mostly, these are the oxidative systems of mixed functions that may contain some metal (usually reduced iron or copper ions), and they were named accordingly: metal catalyzed oxidation (MCO). Within these processes, the reduced metal form (Fe^{2+}, Cu^+) reduces H_2O_2 to hydroxyl radical and phenyl radical (Fenton’s reaction) which thereupon acts on amino acids. Aromatic amino acids are especially sensitive to such oxidation, and in the process oxohistidine or aspartate results from histidine, while carbonyl derivatives (aldehydes or ketones) develop from amino acids, such as lysine, arginine, proline and threonine, which are termed due to their carboxyl groups (88).

A significant oxidant is hypochlorite (HOCl), which is the product of peroxidase reaction. Hypochlorite reacts with tyrosine, tryptophane, lysine and methionine (in protein composition), resulting in chlorotyrosyl, chloramine, aldehyde and methionine sulfoxide, while a reaction with free tyrosine produces p-hydroxy-phenyl-acetaldehyde (pHA), which is covalently bound to lysine residua, thus modifying them (90).

Peroxynitrite, resulting from a reaction of nitric oxide and superoxide anion, most frequently nitrosates the tyrosine residua, as well as cysteine, tryptophane, methionine and phenylalanine residua, and it is a very poor inductor of carbonyl groups (91–94).
γ-rays induce the direct formation of hydroxyl radicals which cause generalized damages to proteins, particularly protein aggregation, degradation and metal-catalyzed oxidation. Accordingly, for example, hydrophobe amino acids: valine, leucin, tyrosine are oxidized in the hydroxy and hydroperoxy derivatives after γ-ray and O2 exposition. The most significant marker of oxidative modification by γ-rays is di-tyrosyl which is at the same time the best, i.e. most sensitive marker of such oxidation (78).

Secondary mechanism of protein oxidative modification

Intracellular proteins may also be oxidized by secondary mechanisms as the result of interaction of free radicals and other cellular constituents such as lipids, hydrocarbons and nucleic acids. Lipid peroxidation and oxidative modification are two independent processes. Their interaction is reflected in the fact that the reactive aldehydes, lipid peroxidation products: MDA, glyoxal and 4-HNE may react with amino groups of proteins forming the intermediaries (36, 95). The most common ligand of these lipid radicals is the ε-amino group of lysine, producing the lipoxidation products, such as N-carboxymethyl lysine (CML), which may also be a glucose derivative. Similar reaction was confirmed on histidine. Such oxidative modification of proteins may occur in plasma lipoprotein metabolism, especially of apoprotein B of the LDL particle, which is extremely significant for the development of atherosclerosis (43).

»Carbonyl stress« is an important form of the secondary oxidation of proteins. Reduced sugars may react non-enzymatically with amino groups of proteins and lipids, through a series of complex biochemical reactions that include oxidative and non-oxidative rearrangement, based on Maillard’s reaction, which finally result in the formation of covalent compounds known as advanced glycosylated end products (AGE-products) (96, 97).

The basic biochemical reaction leading to AGE synthesis proceeds between reduced sugars and proteins by the formation of unstable »Schiff base«. This reaction is fast and reversible. Isomerization of reaction product may be continued via a reaction that is now slower, converting to a ketoamine derivative termed an »Amadori product«. One typical Amadori product is glycosylated hemoglobin (HbA1c), which is used as a classic so-called long-term marker of glycemia in diabetes (i.e., metabolic control marker). This early product may be further involved in complex oxidative reactions of degradation and condensation, where rearrangement occurs within the molecule resulting in AGE products that are now stable and irreversible. These processes may take weeks and months to complete (98). The main substrate of this reaction is glucose, although other sugars may be involved: fructose, threose, glucose-6 phosphate and glyceraldehyde-3 phosphate. The most important AGE products are presented in Table III (Table III).

Synthesis of AGE products begins slowly and goes through several different phases, leading to the damage of protein structure and change of superficial molecule topology, which largely alter the biochemical characteristics of the molecule. The use of specific antibodies against a specific AGE epitope allowed for the detection of AGE in human tissue under physiological conditions, where their concentration tends to rise with aging, showing the association of AGE with degeneration of long-lived proteins and biological aging (99). Theoretically, any protein may become a target of glyco-oxidative modification in vivo. Long-lived proteins including the matrix and structural proteins in the ocular lens and extracellular matrix (ECM) are particularly susceptible to AGE modification. Increased AGE accumulation in tissues may occur due to higher production, lower degradation or longer survival (reduced clearance). Elevated tissue or plasma AGE concentration has been detected in many diseases, such as: DM, chronic renal failure, atherosclerosis, arterial hypertension and Alzheimer’s disease. Moreover, AGE products have been detected in primary rheumatoid arthritis and in the course of osteoporosis (100, 101). Binding of AGE to specific receptors (RAGE) which recognize AGE-modified proteins leads to the activation of intracellular signaling cascades and activation of the entire spectrum of proinflammatory and proinflammatory cellular responses (102). AGE product binding to specific receptors is the major pathogenic factor of complications in diabetes. They are also involved in the pathophysiology of other non-diabetic renal conditions. AGE products are considered to play an important role in glomerular and tubulointerstitial damages, due to the amplification of local immune and inflammatory response of the kidney. Because of impaired renal functioning, AGE products are the major culprits for an abrupt rise in concentration of these products in plasma, which will actually be a vicious circle of disease, deepening the condition even more and aggravating the survival (103–108).

**Table III** Biologically significant AGE

| 1) Monolysyl-products (without cross-link reactions) |
|-----------------------------------------------------|
| a) Nε-carboxy methyl lysine (CML)                    |
| b) Nε-carboxy ethyl lysine (CEL)                     |
| c) Pyralin                                           |
| 2) Bis-lysyl products (cross-linkage)               |
| a) Glyoxal-lysine dimer (GOLD)                      |
| b) Methyl-glyoxal lysine dimer (MOLD)               |
| c) 3-Deoxi-glukazon lysine dimer (DOLD)             |
| 3) Hydroimidazolones                                |
| a) Glyoxal-hydroimidazolone (G-H)                   |
| b) Methylglyoxal-hydroimidazolone (MG-H)            |
| c) 3-deoksiglukazon lysine dimer (3DG-H)            |
| 4) Other cross-linking bonds                         |
| a) Pentosidine                                      |
In diabetes, hyperglycemia triggers the activation of alternative intracellular metabolic pathways (e.g., polyol pathway), wherein the synthesis of these AGE products occurs, and especially: N'-carboxy methyl lysine (CML), N'-carboxy ethyl lysine (CEL), and pentosidine which are the major representatives of this AGE class. In addition, pentosidine is a «cross-linking» molecule that covalently binds distant lysine and arginine residua in proteins, through a C5 ring, thus binding different proteins, together or by forming an intramolecular covalent bond. Alternatively, these products may be generated during fat metabolism, i.e. via oxidation of polyunsaturated fatty acids and arachidonic acid in ion-catalyzed reactions. Products resulting from this type of reaction are called «advanced lipoperoxidation end» (ALE) products. Compounds such as malondialdehyde (MDA)-lysine, hydroxy-nonenal-lysine or acrolein produced by oxidation of hydroxy-amino acids, L-serin or L-threonine, belong to this class of terminally modified proteins. The term AGE is otherwise used for both groups of products. Gluco-oxidative and lipo-oxidative pathways, independent from each other, end in the same final product: CML, while pentosidine develops exclusively from a carbohydrate precursor. Conversely, imidazolone and pyraline may be the result of oxidative stress processes. Non-oxidative compounds may also produce AGE from methylglyoxal during non-oxidative anaerobic glucolysis or on the basis of 3-deoxy-d-arabino-heptulosonate 7-phosphate. Non-oxidative compounds may also produce AGE from methylglyoxal during non-oxidative anaerobic glucolysis or on the basis of 3-deoxy-glycerone that is released from an Amadori rearrangement (109–111).

Diabetes mellitus and uremia are conditions inducing a significant rise of AGE concentrations in tissues and plasma (111). Pentosidine plasma concentration may be even 10 times higher in patients with end-stage renal disease in comparison with healthy controls (112). In diabetics, an increased glucose concentration may be even 10 times higher in patients with end-stage renal disease in comparison with healthy controls (112). In diabetics, an increased glucose level may simply account for a propensity towards the generation of AGE products, by shifting the balance of the biochemical reaction to glycation, i.e. formation of Schiff bases, and irreversible AGE products in accord with the concentration gradient of glucose.

**Determination of oxidatively modified amino acids**

Methodological approaches to the determination of oxidatively modified amino acids are illustrated in Table IV (Table IV).

Tests for the determination of dityrosine, chlorotyrosine and dihydro-phenylalanine are very sensitive and specific; however, the products of tyrosine oxidative modification are elevated in oxidative stress, and are a significant parameter for its determination (113, 114).

Methionine sulfoxide is also a good and sensitive marker of protein oxidative modification. So far, an ELISA assay for the determination of this marker has not been developed in spite of some attempts, because a specific methionine antibody has not been isolated to this day.

Carbonyl products are the most commonly used markers of protein oxidation in biological samples. Carbonyl derivatives of proline, arginine, lysine and threonine are very stable, which facilitates the process of determination and storage. They are present in almost all proteins, in concentrations of approximately 1 nmol/mg of protein, which is equivalent to a concentration of 0.05 mol of carbonyl/mol of protein mass of 50 kDa. In conditions of elevated oxidative stress, their concentration rises 2–8 times in relation to basic values (115). Carbonyl groups may be indu-

| Table IV Methods of determination of oxidatively modified proteins. |
|---------------------------------------------------------------|
| **Modification** | **Detection methods** |
| Disulfides | SDS-gel electrophoresis |
| Thiyl-radicals | electron spin resonance/spectroscopy |
| Glutationylation | HPLC/with mass detection |
| Methionine sulfoxide | cleavage with CNBr/determination of amino acid |
| Carbonyl groups | bonding to DNPH/Western blot/ELISA/HPLC/A370 |
| 2-oxo-histidin | reduction by NaBH3/determination of amino acid |
| Dityrosine | fluorescence: proteolysis or hydrolysis/HPLC |
| Chlorotyrosine | hydrolysis/nitrosanaphthol/HPLC HBr hydrolysis/GH/MD |
| Nitrotyrosine | ELISA; hydrolysis/HPLC; HPLC/electrochemical detection |
| Tryptophanyl | fluorescence; alkaline hydrolysis; proteolysis/MS |
| Hydroperoxides | KI/I3/spectroscopy; NaBH4/hydrolysis/OPA/HPLC |
| Products of lipid peroxidation | NaBH4/hydrolysis/OPA/HPLC GH/MS; DNPH; ELISA |
| Products of glyco-oxidation | derivation/GH/MS |
| Cross-link aggregates | SDS-gel electrophoresis; HPLC |
ced by various types of free radicals both «in vivo» and «in vitro», including the metal-catalyzed oxidation, ozone, HOCl, singlet oxygen and ionizing radiation (113, 116). High-sensitive tests for their detection are available. Many methods include derivation of carbonyl groups with dinitrophenyl hydrazine (DNPH), followed by synthesis of stable dinitrophenyl hydrazone that may be detected by different methods: UV absorption at 370 nm, protein fractionating by HPLC technique, immunochemical ELISA technique, immunohistochemically, one- or two-dimensional SDS-gel electrophoresis with immunoblotting (Western blotting). The last two methods are extremely sensitive and specific (comparing with others), but they are merely semiquantitative.

Advantages and disadvantages of the determination of oxidatively modified proteins

One of the greatest current challenges is the determination of oxidative stress «in vivo». Given that proteins are ubiquitous, i.e. present in all cells and tissues, their determination may serve as a good marker of oxidative stress, considering they are highly susceptible to oxidative stress. Products of protein oxidative modification are determined in different pathological conditions. In some cases, the source of free radicals is known, and sometimes it is not.

In relation to products of lipid peroxidation and DNA oxidative damage, proteins show some advantages:

1. proteins have specific biological functions, therefore, their modification has exceptional functional sequelae (i.e., loss of coagulation function due to fibrinogen oxidative damage or reduced synthesis of ATP due to G3PDH oxidation).

2. Products of protein oxidative damage are relatively stable and sensitive which makes them suitable for determination.

3. The nature of protein modification may provide valid data on the type of oxidant causing the damage:
   a) e.g. chlorotyrosyl is a specific marker of oxidative damage to tyrosine by HOCl action, reflecting most commonly the involvement of neutrophils and monocytes in oxidative stress (117, 118);
   b) nitrotyrosyl residua indicate the presence of increased synthesis of peroxynitrite, i.e. nitric oxide and superoxide (119, 120);
   c) carbonyl groups may be induced by all ROS types, so they may not suggest the type of oxidant, but since they are too difficult to be induced (i.e. they are produced only in extreme conditions of oxidative stress), their presence points to severe oxidative stress-related damage (121);
   d) although methionine and cysteine are most sensitive to oxidative modification, they are not always the markers of altered protein function, because in some cases methionine acts as an internal «scavenger», that is, amino acid protecting the critical acyl residua against oxidative damage (122).

4. High specificity of protein oxidative damage lies in the occasional use of these macromolecules as markers of oxidative damage. There is no «universal marker» of oxidative damage, since there is a large number of causative agents of oxidation as well as various parameters of oxidative damage. Therefore, it would be pointless to determine chlorotyrosyl, and so far neutrophils and monocytes have not been included in the studies of oxidative stress. Singlet oxygen has no effect on carbonyl group induction, but it may oxidize methionine, histidine, tyrosine and tryptophane, so these markers are recommended for the respective process (113).

Oxidative modification of DNA

Free radicals may also bring about the oxidative damages of DNA that are manifested by the development of cancerogenic phenotype if specific proto-oncogenes are involved in a series of degenerative processes, but also in normal processes of aging and apoptosis (123). Although DNA is the most worthy target of action of reactive oxygen species, not all types of radicals are equally toxic and their mode of action is not unique.

The most common types of oxidative modification of the DNA structure are presented in Table V (Table V).

The largest number of DNA damages occur through hydroxyl ion action, while superoxide ion and

| Table V Most common types of DNA oxidative modification. |
|----------------------------------------------------------|
| Break of DNA chain (unilateral or bilateral)             |
| Interaction of bases within one DNA chain spiral         |
| Interaction of bases between two different DNA chain spirales |
| Base change                                              |
| Base loss                                                |
| Base modification                                        |
| Production of pyrimidine dimer                           |
| Generation of 6-4 photoproducts                         |
| Formation of massive obstacles within DNA chain         |
| Interaction with proteins                                |
| Interaction with lipid peroxides (MDA)                  |
| Interaction with glycoxidation products                 |
| Oxidative modification of deoxyribose                   |
hydrogen peroxide are not generally noxious unless Fe^{2+} or Cu^{+} are present, when it comes to a Fenton’s reaction where these free radicals are converted to hydroxyl ion; thereupon, this ion renders multiple modifications of DNA molecules (124, 125). These modifications are most frequently manifested by base loss and formation of abasic sites (AP site), cleavage of DNA chain and sugar modification (126).

AP sites often emerge spontaneously, through depurination. It is estimated that, at least, 10,000 processes of depurination per cell occur within 24 hours under physiological conditions (127).

The double bond between C4-C5 pyrimidine is sensitive to oxidative action of ROS (reactive oxygen species), resulting in a spectrum of pyrimidine derivatives: thymine-glycol, uracil-glycol, urea residue, 5-hydroxy-deoxy-uracil, 5-hydroxy-deoxy-cytosine, while the reaction of free radicals and purines produces the following: 8-hydroxy-2-deoxy guanosine, 8-hydroxy-2-deoxy adenine, form-amino pyrimidine and other less known derivatives (128, 129). Overall changes occurring in a cell are estimated at 200,000 per cell daily. Biological consequences of these modifications are generally known; thymine-glycol blocks DNA replication, which is disastrous for the cell, while 8-oxoguanosine, if not restored by reparatory mechanisms, leads to mutagenesis (130, 131).

Sugar molecule damage as a result of a Fenton’s reaction causes the break of DNA structure and release of base and MDA. OH^- is responsible for Fenton’s reaction causes the break of DNA structure and modification of bases, as well as chromosomal aberrations. UV radiation (254 nm) causes the production of pyrimidine dimers, i.e. formation of a covalent bond between two pyrimidine bases and so-called 6-4 photo-products. Therefore, deletion occurs within a chromosome – formation of dicentric chromosomes and changes in homologue chromosomes. If it is the question of the insufficiency of reparatory system DNA, most commonly the chromosomes are sensitive to UV beams and x-rays (137).

Free radicals may activate the system of poly-ADP-ribosylation, owing to an over-consumption of reduced NADPH at the expense of NADP increase which is a significant stimulus of poly-ribosylation. The rearrangement of genetic material in DNA sequences is especially important, where regulatory genes and proto-oncogenes are coded, because it induces the impairment of cell proliferation, which is one of the signals of cancerogenic phenotype (138).

The cell is protected against toxic DNA damage through a specific localization in the nucleus where free iron as well as superoxide anions can hardly penetrate. Significant protection of DNA structure is provided by histones as well as free radical scavengers which are universal mechanisms of antioxidative defense, either belonging to enzymatic or nonenzymatic antioxidants. Complex mechanisms of DNA reparation imply the presence of adaptation systems of tolerance as well as specific reparatory systems.

Even in the regular process of DNA synthesis, more precisely in polymerization, formation of a wrong base is possible, although mistakes are rare (10^{3}–10^{10} bases). Reparation enzymes are present in conditions of regular functioning and synthesis of DNA. It is the inducible enzymatic system known as »SOS response« or »SOS regulon« which includes a large number of important enzymes, the most significant of which are: DNA endonucleases, AP endonucleases, pyrimidine-hydrate-DNA glycosylase, β-lyase, δ-lyase, deoxy-phospho-diesterase, reparatory 3’ diesterase, DNA polymerases and DNA ligases.

In the human genome, reparation may be completed within only 24 hours if it is exposed to UV rays, where essential genes priority and are repaired in 8 hours.

In a large number of diseases such as Alzheimer’s disease, Huntington’s chorea, Parkinson’s disease, multiple sclerosis, atherosclerosis, etc., reparatory power of DNA is poor, and therefore these patients are hypersensitive to UV rays, radiation or some chemical noxae. Reparatory system insufficiency in the genome is the most important risk factor for a cancerogenic phenotype, especially in conditions of cell exposure to UV rays, gamma radiation or hydrogen peroxide (139, 140).

### Determination of oxidatively modified products of DNA

The most commonly used marker of oxidatively modified DNA molecule is 8-hydroxy-2’-deoxyguanosine (8-OHdG). Biological material most often includes serum or urine, as well as plasma, saliva and tissues. The initial and reference method for this biomarker was HPLC technique. Today, this biomarker...
has been increasingly determined quantitatively by an immunochemical method using monoclonal antibodies (141), or the immunohistochemical method of fluorescent flow cytometry for the detection of oxidatively modified DNA molecules in the composition of cells and tissues (142).

A-purine or a-pyrimidine loci in the DNA molecule are very often sites of damage occurring during base excision and during the repair of oxidatively deaminated and alkylated bases. It is estimated that 20 x 10^5 of these lesions are formed in a cell daily. They are also determined by an immunochemical method termed »ARP assay« (Aldehyde Reactive Probe). Addition of a reagent, containing N-amino-oxymethyl-carbonyl-hydrazine-D-biotin, leads to a specific reaction with the aldehyde group of the damaged base in which the ring is open and the aldehyde group is free. Aldehyde group binds to biotin from ARP reagent, which is then quantified by ELISA testing, using the avidin-biotin complex conjugated with peroxidase or alkaline phosphatase as an indicatory enzyme (143).

The choice of oxidative stress biomarker and method validation

A biomarker of oxidative stress is defined as a biological molecule whose chemical structure is subject to changes, through the action of free radicals, and which may be used reliably for the determination of oxidative stress status in humans and animals. The usefulness of a perfect biomarker of oxidative stress will be shown in its capacity to indicate early onset of the disease or its progression and evaluate the effectiveness of therapy. The measurement of an oxidative stress biomarker may also be of assistance in the elucidation of pathophysiological mechanisms of the oxidative stress-related damage of tissues, in prediction of disease prognosis and selection of adequate treatment in the early stage of disease. A perfect biomarker should be measurable in biological material samples that are easily accessible, such as blood and urine. It would have to possess certain characteristics so as to be a valid biomarker of oxidative stress. It must be:

a) a stable product of oxidative stress, which is not susceptible to artificial induction, oxidation or loss during handling with samples, transport or storage;

b) the key product of oxidative stress, having direct implications for the development and/or progression of disease;

c) detectable in target tissue where it causes oxidative modification and damage;

d) present in sufficient, measurable concentrations;

e) specific for some types of free radicals and its concentrations should not be affected by dietary factors;

f) biological material for testing should be easily obtainable by noninvasive methods and procedures;

g) determinable by methods adequately specific, sensitive and reproducible;

h) relatively easily measurable in the sample of a studied population;

i) its concentration should not vary considerably in the same person, when measured under same conditions but at different times (intraindividual variations); and,

j) have relatively small (acceptable) analytic variation in relation to interindividual (biological) variation.

Validation of an oxidative stress biomarker proceeds through several steps. The first step is analytical validation, implying the development of procedures, analysis of reference material and quality control. The next step is clinical validation, meaning that changes of biomarker concentrations have to correlate with the course of disease, so it can be used with certainty for the prediction of disease progress (144). Determination of reference values and basal values in healthy tissues, cells or body fluids has to be carried out most carefully. The use of a biomarker »panel« may, to a large extent, increase positive predictive test values and reduce the frequency of false-positive and false-negative results. The last step is validation in »epidemiological study«, that is often very difficult to accomplish. Due to the complex development of human diseases associated with oxidative stress, there is an extremely low probability that a single biomarker may fully replace the significance of the clinical outcome. Therefore, development of biomarkers is presently considered the baseline of the better understanding of disease pathogenesis, as well as the process of synthesizing and production of future drugs. The possibility of measurement of biomarkers with the intention of accurate determination of the degree of oxidative damage is of huge importance in clinical trials studying the effectiveness of antioxidant therapy for the prevention and reduction of risk of various complications.
References

1. Commoner B, Townsed J, Pake GE. Free radicals in biological materials. Nature 1954;174: 689–91.

2. Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol 1956; 11: 298–300.

3. Harman D. The aging process. Proc Natl Acad Sci USA 1981; 78: 7124–8.

4. McCord JM, Fridovich I. Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969; 244: 6049–55.

5. Beckman KB, Ames BN. The free radical theory of aging matures. Physiol Rev 1998; 78: 547–81.

6. Mittal CK, Murad F. Activation of guanylate cyclase by superoxide dismutase and hydroxyl radical: a physiological regulator of guanosine 3',5'-monophosphate formation. Proc Natl Acad Sci USA 1977; 74: 4360–4.

7. Ignarro LJ, Kadowitz PJ. The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. Ann Pharmacol Toxicol 1985; 25: 171–91.

8. Radomski MW, Palmer RMJ, Moncada S. The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. Br J Pharmacol 1987; 92: 639–46.

9. Roth S, Dröge W. Regulation of T cell activation and T cell growth factor (TCGF) production by hydrogen peroxide. Cell Immunol 1987; 108: 417–24.

10. Keyse SM, Tyrrell RM. Heme oxygenase in the major physiological role of cyclic GMP in vascular smooth muscle. Ann N Y Acad Sci 1987; 511: 7792–7.

11. Storz G, T artaglia LA, Ames BN. Transcriptional regulation of oxidative stress-inducible genes: direct activation by oxidation. Science 1990; 248:189–94

12. Schreck R, Albermann K, Baeuerle PA. Nuclear factor kappa B: an oxidative stress responsive transcription factor of eucaryotic cells. Free Radic Res Commun 1992; 17: 221–37.

13. Dröge W. Free radicals in the physiological control of cell function. Physiol Rev 2002; 82: 47–95.

14. Čolak E, Majkić-Singh N, Stanković S, Djordjević B Predrag, Srečko-Dimitrijević V, Lalić K, Lalić N. The effect of hyperglycemia on the values of antioxidative parameters in type 2 diabetic patients with cardiovascular complications. Jugoslov Med Biophys Acta 1995; 1271: 63–6.

15. Čolak E, Majkić-Singh N, Stanković S, Srečko-Dimitrijević V, Đorđević PB, Lalić K, Lalić N. Parameters of antioxidative defense in type 2 diabetic patients with cardiovascular complications. Ann Med 2005; 37: 613–20.

16. Đorđević VB, Pavlović DD, Kocić GM. Biohemija slobodnih radikalova, Niš 2000.

17. Halliwell B, Gutteridge JMC. Lipid peroxidation: a radical chain reaction. 1985; 189–93. In: Free radicals in biology and medicine, Oxford: Clarendon Press, 1985.

18. Karayalcin SS, Sturbaum CW, Wachman JT, Cha JH, Powell DW. Hydrogen peroxide stimulates rat colonic prostaglandin production and alters electrolyte transport. J Clin Investig 1990; 86: 60–8.

19. Korsmeyer SJ, Yin XM, Oltvai ZN, Veis-Novack DJ, Linette GP. Reactive oxygen species and the regulation of cell death by the Bcl-2 gene family. Biochim Biophys Acta 1995; 1271: 63–6.

20. Kagan VE. Lipid peroxidation in biologoumnes. Florida: CRC Press, Inc. Boca Raton 1988.

21. Aust SD, Morehouse LA, Thomas CE. Role of metals in oxygen radical reactions. J Free Radic Biol Med 1985; 1: 3–25.

22. Fong KL, McCay PB, Poyer JL, Kerle BB, Misra H. Evidence that peroxidation of lysosomal membranes is initiated by hydroxyl free radicals, produced during flavin enzyme activity. J Biol Chem 1975; 248: 7792–7.

23. Gargner HW. Oxygen radical chemistry of polysaturated fatty acids. Free Radic Biol Med 1989; 7: 65–86.

24. Girotti AW. Lipid hydroperoxide generation turnover, and effector action in biological systems. J Lipid Res 1998; 59: 1529–42.

25. Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative damage in human disease. Clin Chem 2006; 52: 601–23.

26. Stocker R, Keaney JF. Jr. Role of oxidative modifications in atherosclerosis. Physiol Rev 2004; 84: 1381–478.

27. Berliner J, Heinicke JW. The role of oxidized lipoproteins in atherogenesis. Free Radic Biol Med 1996; 20: 707–27.

28. Slatter DA, Bolton CH, Bailey AJ. The importance of lipid-derived malondialdehyde in diabetes mellitus. Diabetologia 2000; 43: 550–7.

29. Heinicke JW. Oxidized amino acids: culprits in human atherosclerosis and indicators of oxidative stress. Free Radic Biol Med 2002; 32: 1090–101.

30. Yoneyama Y, Sawa R, Suzuki S, Doi D, Yoneyama K, Otsubo Y, et al. Relationship between plasma malondialdehyde levels and adenosine deaminase activities in preeclampsia. Clin Chim Acta 2002; 322: 169–73.

31. Wood LG, Gibson PG, Garg ML. Biomarkers of lipid peroxidation, airway inflammation and asthma. Eur Respir J 2003; 21: 177–86.

32. Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov 2004; 3: 205–14.

33. Uchida K. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. Prog Lipid Res 2003; 42: 318–43.
49. Pryor WA, Stanely JP, Blair E. Autoxidation of polyunsaturated fatty acids. A suggested mechanism for the formation of TBA-reactive materials from prostaglandin-like endoperoxides. Lipids 1976; 11: 370–9.

50. Morrow JD, Hill KE, Burk RF, Nammour TM, Badr KF, Roberts LJ II. A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. Proc Natl Acad Sci USA 1990; 87: 9383–7.

51. Taber DF, Morrow JD, Roberts LJ II. A nomenclature system for the isoprostanes. Prostaglandins 1997; 53: 63–7.

52. Morrow JD, Awad JA, Boss HJ, Blair IA, Roberts LJ II. Non-cyclooxygenase-derived prostanooids (F2-isoprostanes) are formed in situ on phospholipids. Proc Natl Acad Sci USA 1989; 86: 10721–5.

53. Morrow JD, Roberts LJ II. The isoprostanes: unique bioactive products of lipid peroxidation. Prog Lipid Res. 1997; 36: 1–21.

54. Chen Y, Morrow JD, Roberts LJ II. Formation of reactive cyclopentenone compounds in vivo as products of the isoprostane pathway. J Biol Chem 1999; 274: 10863–8.

55. Brune K, Salomon RG, Morrow JD, Roberts LJ II. Identification of extremely reactive \( \gamma \)-ketoaddehydes (isolevuglandins) as products of the isoprostane pathway and characterization of their lysyl protein adducts. J Biol Chem 1999; 274: 13139–46.

56. Roberts LJ II, Moore KP, Zacker WT, Oates JA, Morrow JD. Identification of the major urinary metabolite of the F2-isoprostaglandin F2 in humans. J Biol Chem 1996; 271: 20617–20.

57. Roberts LJ II, Montine TJ, Markesbery WR, Tapper AR, Hardy P, Chembob S, et al. Formation of isoprostane-like compounds (neuroprostanes) in vivo from docosahexaenoic acid. J Biol Chem 1998; 273: 13605–12.

58. Marnett LJ. Lipid peroxidation–DNA damage by malondialdehyde. Mutat Res 1999; 424: 83–95.

59. Wang M, Dhirgra K, Hittleman WN, Liehr JG, De Andrade M, Li D. Lipid peroxidation-induced putative malondialdehyde–DNA adducts in human breast tissues. Cancer Epidemiol Biomarkers Prev. 1996; 5: 705–10.

60. Orioli M, Aldini G, Beretta G, Facino RM, Carini M. LC-ESI-MS/MS determination of 4-hydroxy-trans-2-nonenal Michael adducts with cysteine and histidine-containing peptides as early markers of oxidative stress in excitable tissues. J Chromatogr B Analyt Technol Biomed Life Sci 2005; 827 (1): 109–18

61. Aldini G, Granata P, Carini M. Detoxification of cytotoxic alpha, beta-unsaturated aldehydes by carnosine: characterisation of conjugated adducts by electrospray ionization tandem mass spectrometry and detection by liquid chromatography/mass spectrometry in rat skeletal muscle. J Mass Spectrom 2002; 37 (12): 1219–28.

62. Borović S, Rabuzin F, Waeg G, Žarković N. Enzyme-linked immunosorbent assay for 4-hydroxy-nonenal–histidine conjugates. Free Radic Res 2006; 40 (8): 809–20.
63. Carini M, Aldini G, Beretta G, Arlandini E, Facino RM. Acronein-sequestering ability of endogenous dipeptides: characterization of carnosine and homocarnosine/acronein adducts by electrospray ionization tandem mass spectrometry. J Mass Spectrom 2003; 38 (9): 996–1006.

64. Zhang S, Villalta PW, Wang M, Hecht SS. Detection and quantitation of acronein-derived 1,N2-propanodeoxyguanosine adducts in human lung by liquid chromatography-electrospray ionization-tandem mass spectrometry. Chem Res Toxicol 2007; 20 (4): 565–71.

65. Pratico D, Lawson JA, Rokach J, FitzGerald KA. The isoprostanes in biology and medicine. Trends Endocrinol. Metab 2001; 12: 243–7.

66. Wang Z, Ciabattoni G, Creminon C, Lawson J, FitzGerald KA, Patrono C, et al. Immunological characterization of urinary 8-epi-prostaglandin F2α excretion in man. J Pharmacol Exp Ther 1995; 275: 94–100.

67. Proudfoot J, Barden A, Mori TA, Burke V, Croft KD, Beillin LJ, et al. Measurement of urinary F2-isoprostanes as markers of in vivo lipid peroxidation. A comparison of enzyme immunoassay with gas chromatography/mass spectrometry. Anal Biochem 1999; 272: 209–15.

68. Gopaul NK, Halliwell B, Anggard EE. Measurement of plasma F2-isoprostanes as an index of lipid peroxidation does not appear to be confounded by diet. Free Radic Res 2000; 35: 115–27.

69. Gopaul NK, Zacharowski K, Halliwell B, Anggard EE. Evaluation of the postprandial effects of a fast-food meal on human plasma F2-isoprostane levels. Free Radic Biol Med 2000; 28: 806–14.

70. Cracowski JL, Durand T, Bessard G. Isoprostanes as a biomarker of lipid peroxidation in humans: physiologic, pharmacology and clinical implications. Trends Pharmacol Sci 2002; 23: 360–6.

71. Helmersson J, Basu S. F2-Isoprostane excretion rate and diurnal variation in human urine. Prostaglandins Leukot Essent Fatty Acids 1999; 61 203–5.

72. Montuschi P. Indirect monitoring of lung inflammation. Nat Rev Drug Discov 2002; 1: 238–42.

73. Montuschi P, Barnes PJ. Analysis of exhaled breath condensate for monitoring airway inflammation. Trends Pharmacol Sci 2002; 23: 252–7.

74. Lynch SM, Morrow JD, Roberts LJ II, Frei B. Formation of non-cyclooxygenase-derived prostanooids (F2-isoprostanes) in plasma and low density lipoprotein exposed to oxidative stress in vitro. J Clin Invest 1994; 93: 998–1004.

75. Stadtman ER. Protein oxidation and aging. Free Radic Res 2006; 40 (12): 1250–8.

76. Stadtman ER. Role of oxidant species in aging. Curr Med Chem 2004; 11 (9): 1105–12.

77. Miyoshi N, Oubrahim H, Chock PB, Stadtman ER. Age-dependent cell death and the role of ATP in hydrogen peroxide-induced apoptosis and necrosis. Proc Natl Acad Sci USA 2006; 103 (6): 1727–31.

78. Requena JR, Levine RL, Stadtman ER. Recent advances in the analysis of oxidized proteins. Amino Acids 2003; (3–4): 221–6.

79. Van der Vliet A, Nguyen MN, Shigenaga MK, Eiserich JP, Marelich GP, Cross CE. Myeloperoxidase and protein oxidation in cystic fibrosis. Am J Physiol 2000; 279: L537–46.

80. Bota DA, Davies KJ. Protein degradation in mitochondria: implications for oxidative stress, aging and disease: a novel etiological classification of mitochondrial proteolytic disorders. Mitochondrion 2001; (1): 33–49.

81. Refsgaard HH, Tsai L, Stadtman ER. Modifications of proteins by polyunsaturated fatty acid peroxidation products. Proc Natl Acad Sci USA 2000; 97 (2): 611–6.

82. Stadtman ER, Levine RL. Oxidation of cellular proteins by beta-amyloid peptide. Neurobiol Aging 1999, 3: 351–42.

83. Stadtman ER, Levine RL. Free radical-mediated oxidation of free amino acids and amino acids residues in proteins. Amino Acids 2003; 3–4: 207–18.

84. Stadtman ER, Moskovitz J, Levine RL. Oxidation of methionine residues in proteins: biological consequences. Antioxid Redox Signal 2003; 5: 577–82.

85. Stadtman ER, Van Rommer H, Richardson A, Wehr NB, Levine RL. Methionine oxidation and aging. Biochim Biophys Acta 2005; 1703 (2): 135–40.

86. Dalle-Donne I, Scaloni A, Giustarini D, Cavarra E, Tel1 G, Lungarella G, et al. Proteins as biomarkers of oxidative stress in diseases: the contribution of redox proteomics. Mass Spectrom Rev 2005; 24: 55–99.

87. Davies MJ, Fu S, Wang H, Dean RT. Stable markers of oxidant damage to proteins and their application in study of human disease. Free Radic Biol Med 1999; 27: 1151–61.

88. Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milanzi A. Protein carbonylation in human diseases. Trends Mol Med 2003; 9: 169–76.

89. Giulini C, Traaseth NJ, Davies KJ. Tyrosine oxidation products: analysis and biological relevance. Amino Acids 2003; (3–4): 227–32.

90. Koppenol WH, Moreno JJ, Pryor WA, Ischiropoulos H, Beckman JS. Peroxynitrite: a cloaked oxidant from superoxide and nitric oxide. Chem Res Toxicol 1992; 5: 834–42.

91. Richeson CE, Mulder P, Bowry VW, Ingold KU. The complex chemistry of peroxynitrite decomposition: new insights. J Am Chem Soc 1998; 120: 7211–9.

92. Dedon PC, Tannenbaum SR. Reactive nitrogen species in the chemical biology of inflammation. Arch Biochem Biophys 2004; 423: 12–22.

93. Ischiropoulos H. Biological selectivity and functional aspects of protein tyrosine nitration. Biochem Biophys Res Commun 2003; 305: 776–83.

94. Requena JR, Levine RL, Stadtman ER. Recent advances in the analysis of oxidized proteins. Amino Acids 2003; 25: 221–6.
95. Bohlender MJ, Franke S, Stein, Wolf G. Advanced glycation end products and the kidney. Am J Physiol Renal Physiol 2005; 289: 643–59.

96. Yamagishi S, Nakamura K, Imaiizumi T. Advanced glycation end products (AGEs) and diabetic vascular complications. Curr Diabet Rev 2005; 1: 93–106.

97. Dyer DG, Dun JA, Thorpe SR, Baleie KE, Lyons TJ, McCance DR, et al. Accumulation of Maillard reaction products in skin collagen in diabetes and aging. J Clin Invest 1995; 91: 2463–9.

98. Hein G, Wiegand R, Lehmann G, Stein G, and Franke S. Advanced glycation end-products pentosidine and N-carboxymethyllysine are elevated in serum of patients with osteoporosis. Rheumatology 2003; 42: 1242–6.

99. Suzuki D, Miyata T, Saotome N, Horie K, Inagi R, Yasuda Y, et al. Immunohistochemical evidence for an increased oxidative stress and carbonyl modification of proteins in diabetic glomerular lesion. J Am Soc Nephrol 1999; 10: 822–32.

100. Schmidt AM, Vianna M, Gerlach M, Brett J, Ryan J, Kao J, et al. Isolation and characterization of binding proteins for advanced glycosylation end products from lung tissue which are present on the endothelial surface. J Biol Chem 1992; 267: 14987–97.

101. Aronson D. Cross-linking of glycated collagen in the pathogenesis of arterial and myocardial stiffening. J Hypertens 2003; 21: 3–12.

102. Miyata T, Kurokawa K, Van Ypersele de Strihou C. Advanced glycation and lipoxidation end products: role of reactive carbonyl compounds generated during carbohydrate and lipid metabolism. J Am Soc Nephrol 2000; 11: 1744–52.

103. Schmidt AM, Yan SD, Yan SF, Stern DM. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. J Clin Invest 2001; 108: 949–55

104. Singh R, Bardou A, Mori T, Beilin L. Advanced glycation end-products: a review. Diabetologia 2001; 44: 129–46.

105. Wautier JL, Schmidt AM. Protein glycation: a firm link to endothelial cell dysfunction. Circ Res 2004; 95: 233–8.

106. Wautier JL, Schmidt AM. Protein glycation: a firm link to endothelial cell dysfunction. Circ Res 2004; 95: 233–8.

107. Miyata T, Fu MX, Kurokawa K, Van Ypersele de Strihou C, Thorpe SR, Baynes JW. Autoxidation products of both carbohydrates and lipids are increased in uremic plasma: is there oxidative stress in uremia? Kidney Int 1998; 54: 1290–5.

108. Miyata T, Van Ypersele de Strihou C, Kurokawa K, Baynes JW. Alterations in non-enzymatic biochemistry in uremia: origin and significance of «carbonyl stress» in long-term uremic complications. Kidney Int 1999; 55: 389–99.

109. Niwa T, Katsuzaki T, Momoi T, Miyazaki T, Ogawa H, Saito A, et al. Modification of 2m with advanced glycation end products as observed in dialysis related amyloidosis by 3-DG accumulating in uremic serum. Kidney Int 1996; 49: 861–7.

110. Makita Z, Radoff S, Rayfield EJ, Yang Z, Skolnik E, Delaney V, et al. Advanced glycosylation end products in patients with diabetic nephropathy. N Engl J Med 1991; 325: 836–42.

111. Miyata T, Ueda Y, Shinzato T, Iida Y, Tanaka S, Kurokawa K, et al. Accumulation of albumin-linked and free-form pentosidine in the circulation of uremic patients with end-stage renal failure: renal implications in the pathophysiology of pentosidine. J Am Soc Nephrol 1996; 7: 1198–206.

112. Shacter E. Quantification and significance of protein oxidation in biological samples. Drug Metab Rev 2000; 32 (3–4): 307–26.

113. Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. Biochem J 1997; 324: 1–18.

114. Stadtman ER, Berlett BS. Reactive oxygen mediated protein oxidation in aging and disease. Drug Metab Rev 1998; 30: 225–43.

115. Yan LJ, Lodge JK, Traber MG, Matsugo S, Packer L. Comparison between copper-mediated and hypochlorite-mediated modification of human low density lipoproteins evaluated by protein carbonyl formation. J Lipid Res 1997; 38: 992–1001.

116. Gaut JP, Yeh GC, Tran HD, Byun J, Henderson JP, Richter GM, et al. Neutrophils employ the myeloperoxidase system to generate antimicrobial brominating and chlorinating oxidants during sepsis. Proc Natl Acad Sci U S A 2001; 98: 11961–6.

117. Hazen SL, Heinecke JW. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. J Clin Invest 1997; 99: 2075–81.

118. Duncan MW. A review of approaches to the analysis of 3-nitrotyrosine. Amino Acids 2003; 25: 351–61.

119. Tsikas D, Mitschke A, Suchy MT, Gutzk FM, Stichentoth DO. Determination of 3-nitrotyrosine in human urine at the basal state by gas chromatography-tandem mass spectrometry and evaluation of the excretion after oral intake. J Chromatogr B 2005; 827: 146–56.

120. Levine RL, Wehr N, Williams JA, Stadtman ER, Shacter E. Determination of carbonyl groups in oxidized proteins. Methods Mol Biol 2000; 99: 15–24.

121. Stadtman ER, Moskovitz J, Berlett BS, Richter GM. Cyclic oxidation and reduction of protein methionine residues is an important antioxidant mechanism. Mol Cell Biochem 2002; 234–235 (1–2): 3–9.

122. Ames BN. Endogenous DNA damage as related to aging and mortality. Proc Natl Acad Sci U S A 1991; 88: 1853–7.

123. Cadet J, Delatour T, Douki T, Gasparutto D, Pouget JP, Ravanat JL, et al. Hydroxyl radicals and DNA base damage. Mutat Res Fundam Mol Mech Mutagen 1999; 424: 9–21.
124. Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. Curr Med Chem 2005; 12 (10): 1161–208.
125. Henle ES, Linn S. Formation, prevention and repair of DNA damage by iron/hydrogen peroxide. J Biol Chem 1997; 272: 19095–98.
126. Beckman KB, Ames BN. Endogenous oxidative damage of mtDNA. Mutat Res 1999; 424: 51–8.
127. Wang D, Kreutzer DA, Essigmann JM. Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions. Mutat Res Fundam Mol Mech Mutagen 1998; 400: 99–115.
128. Kreutzer DA, Essigmann JM. Oxidized, deaminated cytosines are a sources of C T transitions in vivo. Proc Natl Acad Sci USA, 1998; 95: 3578–82.
129. Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. B-hydroxyguanine, an abundant form of oxidative DNA damage, causes G T and A C substitutions. J. Biol Chem 1992; 267: 166–72.
130. Le XC, Xing JZ, Lee J, Leandon SA, Weinfeld M. Inducible repair of thymine glycol detected by an ultrasensitive assay for DNA damage. Science 1998; 280: 1066–9.
131. Yau TM. Mutagenicity and cytotoxicity of malondialdehyde in mammalian cells. Mech Ageing Dev 1979; 11: 137–44.
132. Kocić G, Pavlović D, Đorđević VB, Bjelaković G, Stojanović I. Role of nitric oxide and peroxynitrite in apoptosis relation to endonuclease activity. Jugoslov Med Biohem 2003; 22 (2): 95–100.
133. Marnett LJ. Lipid peroxidation-DNA damage by malondialdehyde. Mutat Res 1999; 424: 83–95.
134. Wallace SS. Enzymatic processing of radiation-induced free radical damage in DNA. Radiat Res 1998; 150: 560–79.
135. Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H. Free radical-induced damage to DNA: mechanisms and measurement. Free Radic Biol Med 2002; 32: 1102–15.
136. Halliwell B. Effect of diet on cancer development: is oxidative DNA damage a biomarker? Free Radic Biol Med 2002; 32: 968–74.
137. Malins DC, Haimanot R. Major alterations in the nucleotide structure of DNA in cancer of the female breast. Cancer Res 1991; 51: 5430–2.
138. Nair J, Carmichael PL, Fernando RC, Phillips DH, Strain AJ, Bartsch H. Lipid peroxidation-induced etheno-DNA adducts in the liver of patients with the genetic metal storage disorders Wilson’s disease and primary hemochromatosis. Cancer Epidemiol Biomarkers Prev 1998; 7: 435–40.
139. Poulsen HE, Loft S, Prieme H, Vistisen K, Lykkesfeldt J, Nyyssonen K, et al. Oxidative DNA damage in vivo: relationship to age, plasma antioxidants, drug metabolism, glutathione-S-transferase activity and urinary creatinine excretion. Free Radic Res 1998; 29: 565–71.
140. http://www.assaydesigns.com.
141. http://www.kamiyabiomedical.com.
142. http://www.dojindo.com.newsletter/review_vol2.htm.
143. Čolak E, Srečković-Dimitrijević V, Đorđević PB, Stanković S, Majkić-Singh N, Lalić K, et al. The influence of type and duration of cardiovascular complications on antioxidative parameter values in type 2 diabetic patients. JMB 2007; 26 (1): 10–6.

Received: September 20, 2007
Accepted: November 10, 2007