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ROLE OF FREE RADICALS IN OXIDATIVE STRESS – BASIC KNOWLEDGE FOR CLINICIAN

Oxygen free radicals are thought to be involved in pathogenesis of various diseases in humans and animals. Living organisms have diverse defense mechanisms, both enzymatic and non-enzymatic. The aim of this review is state-of the art description of the role of reactive oxygen species on oxidative stress development in living organism.

Oxidative stress is most simply defined as an imbalance between oxidants and antioxidants in which the oxidant activity exceeds the neutralizing capability of antioxidants, resulting in cellular injury and activation of pathologic pathways. Within this context, the oxidants of interest are collectively referred to as reactive oxygen species, which can be defined as oxygen-containing molecules that are more reactive than the triplet oxygen molecules present in air. The biologically relevant molecules meeting this criterion include the superoxide anion radical, perhydroxyl radical, hydroxyl radical, and hydrogen peroxide. The human and animal body is well equipped to deal with the production of these molecules with endogenous antioxidant scavenging systems, which include antioxidant enzymes as well as, nonenzymatic antioxidants. The field of oxidative stress research and evidence-based antioxidant therapy in human and animal medicine is still in the early stages of development. There is a great deal to be discovered about the importance and basic pathophysiology of oxidative stress in living organism. Even as oxidant injury is proven to be associated with numerous conditions, it still remains to be seen if it is a primary cause of pathologic change or a secondary effect of disease.

Key words: ROS, Oxidative stress, Free oxygen radical, Antioxidants.

Introduction. In recent years there have been a growing number of studies on disturbance of balance between the formation of reactive oxygen species (ROS) and the antioxidant efficiency of the body. Numerous studies confirm involvement of ROS in aetiopathogenesis of a number of diseases as well as describe the intensification of free radical generation in the course of physical exertion [13, 25]. Such studies concentrate mostly on humans and laboratory animals [6, 18].

Oxidative stress is defined as increased generation of reactive oxygen species, exceeding the capacity of physiological antioxidative systems. Those conditions reveal the damaging impact of the reactive oxygen species on organic compounds, in particular lipids and proteins, which results in structural and functional disorders at the molecular and cellular levels. Such situation can occur both under the conditions of increased rate of endogenous ROS production, exposure to additional inducing factors, and impairment of natural antioxidative mechanisms of the organism [3, 7].

Formations of reactive oxygen species (ROS). From the biological point of view, the most important compounds causing oxidative damage of cellular structures are such ROS's as: superoxide anion radical ($O_2^{\bullet-}$), hydroxylic radical ($OH^{\bullet}$), hydroperoxide radical ($HO_2^{\bullet}$), peroxide radical ($ROO^{\bullet}$), as well as compounds which are not free radicals, but exhibit similar action, such as: hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) [9, 40]. The main precursor of most free radical reactions is superoxide anion radical, which is a free radical with one unpaired electron. It is created as a result of a single electrode reduction of molecular oxygen [4]:

$$O_2 + e^- \rightarrow O_2^{\bullet-}$$

The presence of a superoxide anion radical in the cell very quickly leads to the formation of next reactive oxygen species. Compared to oxygen, $O_2^{\bullet-}$ reacts with a greater number of substances and, as a rule, does it faster. The radical reacts very non-specifically and can react with practically any substances present in the body [36]. The reactions of the superoxide anion radical, as well as the hydroxyl radical, are characterised by small specificity, and consequently it is almost certain that they will react with first molecule they come across [5].

Formation of reactive oxygen species in the organism can increase as a result of external factors, such as ionizing radiation, the impact of sunlight, or ultrasounds, as well as internal factors, such as some diseases, physical exertion, stress, inappropriate diet, and aging processes of the organism [19, 51]. From the biological point of view, the most important producer of free radicals is the mitochon-

drial respiratory chain [1]. The amount of ROS's in the organism is subject to very dynamic changes and is a product of the processes of formation and deactivation of free radicals. In normally functioning cells there area certain ROS concentrations, constant over time, which are a product of that dynamic balance. Such balance is disrupted by the situations described above. Free radicals are generated in some organs at a much faster rate than in the other organs, and their production is particularly intensified in the liver, the heart, skeletal muscles, and the brain [16].

Antioxidant compensatory mechanisms. In the course of evolution aerobic organisms, because of the consequences of free radical threats related to the use of oxygen, developed a number of defence mechanisms [11]. The role of biological antioxidants in organisms is fulfilled by specialised enzymes as well as non-enzymatic molecules, such as low-molecular antioxidants. Such compounds are present in living organisms in much smaller quantities than the oxidizable substrate.

The main tasks of an efficient antioxidative protection system are to prevent the formation of ROS's and their reaction with cell components, stop free radical chain reactions, and rectify the consequences of ROS reactions with biomolecules [14].

The first of the above tasks is performed by the so-called enzymatic triad, which consists of disproportionating enzymes, i.e. catalase (CAT), superoxide dysmutase (SOD), glutathione peroxidase (GPx), and proteins binding ions of transition metals [10]. The execution of the second task is mostly the responsibility of low-molecular antioxidants, i.e. "scavengers" of free radicals. The last defence strategy involves rectification of the results of ROS reaction with cellular macromolecules, e.g. by elimination of damaged proteins or repair of nucleic acids [15, 20].

Superoxide dysmutase is an enzyme which catalyzes the reaction of disproportionation of superoxide anion radical [32]. The enzyme is a metalloprotein consisting of the protein part and the catalytic prosthetic group in the form of an atom of metal, acting as the active centre. The reaction of dismutation of superoxide anion radical is accompanied by the formation of hydrogen peroxide and oxygen molecule.

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

In mammal organisms there are three dysmutase isoenzymes: cytoplasmic (Cu/ZnSOD-1), mitochondrial (MnSOD-2), and secreted outside the cell, present in the lymph, the plasma, and the synovial fluid (EC-SOD-3) [49]. The first isoenzyme is primarily present in the cytoplasm and the nucleus, whereas the second isoenzyme is located primarily in the mitochondrial matrix. Outside the cells, mainly on their surface, there is extracellular superoxide dysmutase connected with proteoglycans [17, 24]. Its molecules consist of four subunits and contain saccharide residues. EC-SOD is a protein structurally similar to MnSOD, but containing Cu and Zn in the catalytic centre. Binding of that enzyme to the cell surface can be an effective method of protection of cells against superoxide anion radical generated in the external environment [33].

Hydrogen peroxide formed as a result of dysmutase activity is then decomposed by glutathione peroxidase (GPx) and catalase. GPx is responsible for catabolism of the majority of hydrogen peroxide formed in the cells [29, 35]. It has greater affinity to hydrogen peroxide than catalase, which demonstrates its special role in the removal of that compound when its concentration in the cell is low. In the case of high substrate concentration its role is taken over by catalase.

Glutathione peroxidase, an enzyme whose structure contains selenium, catalyzes reaction between reduced glutathione (GSH) and hydrogen peroxide, resulting in the formation of oxidized form of glutathione, i.e. glutathione disulfide (GSSG) and water [39].

$$2GSH + H_2O_2 \rightarrow GSSG + H_2O$$

Due to the fact that glutathione disulfide is a dangerous compound for cells as it can enter into subsequent reactions with proteins, the above reaction is connected with glutathione reductase, i.e. an enzyme forming an additional amount of the reduced form of glutathione. That reaction takes place at the cost of NADPH oxidation [39, 45].

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

The second, immensely important enzyme catalysing distribution of hydrogen peroxide molecules is catalase (CAT). That enzyme is a hemoprotein, whose structure contains 4 heme groups [37]. In the cells of eukaryotic organisms catalase is located mainly in peroxisomes, where it is accompanied by other oxire-

ductase enzymes. The biggest activity of the enzyme has been demonstrated in liver, erythrocytes, bone marrow, kidneys, and mucous membranes, whereas the least activity was detected in the connective tissue [2]. CAT activity depends on the substrate concentration, the temperature, pH, and the presence of activators and inhibitors. Enzymes of eukaryotic organisms are active in the broad pH range (5.0–10.5); however, the optimum pH for catalase is thought to be pH 7.0. The discussed enzyme is characterised by a very high operation speed. One molecule dissolves in one second approx. 200,000 molecules of hydrogen peroxide. The reaction is accompanied by the formation of water and oxygen [26, 31]:

$$2\ H_2O_2 \rightarrow 2H_2O + O_2$$

In the case of many diseases there is a noticeable weakening of catalase activity, especially the diseases whose pathophysiology is explained by oxidative stress.

An important role in the protection of living organisms against the harmful effect of free radical reactions is played by low-molecular antioxidants. Basically, the reactions of low-molecular antioxidants are characterised by lower specificity compared to the already described enzymatic triad, but it provides the organisms with a more universal and comprehensive protection.

In the cell environment antioxidants are distributed both in the aqueous phase and in the lipid phase. Water-soluble hydrophilic compounds can be found mostly in the cytoplasm and some cell organelles, whereas hydrophobic compounds can be found in the lipid layer of cell membranes.

The most important water-soluble antioxidants are ascorbic acid (vitamin C), glutathione, uric acid, creatinine, cysteine, bilirubin, and albumins [41].

The most effective antioxidant of the extracellular space is thought to be ascorbic acid. Under physiological conditions, Vitamin C is present in a partially-ionized form as ascorbate. Plants and most animals are capable of synthesising vitamin C, but guinea pigs, bats, people, and other primates must receive vitamin C in food. This is caused by loss of an enzyme – gluconolactone oxidase, which takes place in the final stage of vitamin C biosynthesis [23]. Strong reduction properties of ascorbate enable its reactions with hydrogen peroxide, superoxide anion radical, hydroxyl radical, singlet oxygen, and chloric acid (I). Oxidation of ascorbate leads to the formation of a relatively low-reactive L-ascorbic acid radical and dehydroascorbate, followed by the formation of oxalates and other oxidation products. It is believed that ascorbate, too, can directly contribute to the prevention of lipid peroxidation through reduction of the oxidised form of α- tocopherol. The above properties are exhibited by ascorbate at high concentration levels, whereas in low amounts it exhibits prooxidative properties. Such activity is revealed only in the presence of ions of transition metals, in particular Fe^{3+}, thus leading to the initiation of the Fenton reaction [12, 21].

One of the most important low-molecular antioxidants is the reduced glutathione [34]. Its properties are manifested in reproduction of the -SH thiol groups in proteins, where they were oxidised to the $-SO_3H$ sulphonic groups or the -S-S- disulfide bridges. Thanks to the reversible reaction of electron detachment or attachment, glutathione acts in the organism as an oxidation-reduction system protecting the –SH groups of proteins against oxidation [28, 31]. In red blood cells the reduced glutathione (GSH) acts like a hydrosulphide buffer at a concentration of approx. 5 $mmol \cdot L^{-1}$, maintaining cysteine residues of haemoglobin and other proteins in reduced form [44].

Another important antioxidant present in people and humans is uric acid, one of the end products of purine transition [50]. It fulfils two functions in the antioxidative defence – on the one hand, it binds ions of the Fe^{2+} iron, on the other hand, it react with oxidants, which leads to the formation of uric anion radical. The end product of the described reaction is allantoin. Both those products are characterised by relatively low reactivity towards oxygen and cell components. Large amounts of uric acid are present primarily in the endothelium of the blood vessels, in the mucous membrane of the intestines, and in the liver [38, 43].

An important role in the functioning of the cell and the whole organism is also played by hydrophobic antioxidants, such as tocopherols and carotenoids [47]. They operate primarily in the lipid two layers of cell membranes. Their role is especially important because this is the place where the process of lipid peroxidation takes place.

A special role in the trapping of free radicals is played by blood plasma proteins, and in particular albumins. Those proteins, due to their significant number of thiol groups, exhibit high reactivity with free radicals. Albumins can also bind ions of copper, thus protecting fatty acids against copper-

dependent oxidation processes. This is especially important in the context of albumins as proteins transporting free fatty acids in the blood serum [52].

Strong antioxidative properties are also exhibited by bilirubin. That compound, by reacting with peroxide radicals and singlet oxygen, protects linolenic acid against oxidation. Research on people has demonstrated a relationship between increased bilirubin concentration in the blood serum and lower incidence of cardiovascular diseases in men [8]. It is also believed that hyperbilirubinemia present in infants, while potentially toxic in certain situations, probably constitutes an effective defence mechanism against oxidation processes related to the first contact with the air oxygen [30, 42].

Vitamin E (tocopherol) is considered one of the strongest lipophilic antioxidants present in cell membranes and plasma lipoproteins. There are several varieties of tocopherols (alpha, beta, gamma, delta), of which the highest biological activity is exhibited by alpha-tocopherol. Its antioxidative activity involves inhibition of oxidation of polysaturated fatty acids through the breaking of chain reactions, generating free peroxide radicals of the oxidised acid. By removing secondary free radicals, tocopherols ensure structural and functional integrity of cell membranes and intracellular organelles [46].

Tocopherols react with peroxide radicals forming in biological membranes and lipoproteins, creating relatively stable tocopherol radicals. Those radicals do not take part in the next stage in the prolongation chain of free radical reactions, but are reduced by other antioxidants. Reactions can also involve two tocopherol radicals, terminating two sequences of free radical reactions [22].

Another group of hydrophobic antioxidants are carotenoids. From the chemical point of view, one distinctive feature of carotenoids is the presence of two cyclohexylamine rings connected by a long carbon chain, which contains a system of coupled carbon-carbon double bonds. And it is this bond that gives antioxidative carotenoids their distinctive features, i.e. high activity towards singlet oxygen and organic radicals, which are a derivative of the process of lipid peroxidation. The reaction of addition of lipid peroxide radical to β-carotene results in a carotenoid free radical, which then reacts with another lipid peroxide [27, 48].

Conclusion. The area of oxidative stress research in human and proven acting of antioxidant therapy in medicine is still in the early stages of development. There is a great chance to discover the influence and basic pathophysiology of oxidative stress in humans and companion animals. Even as oxidative stress is proven to be associated with diseases, it still remains to be discovered, if it is a primary cause of a harm or a consequence of sickness processes. The future research will better show the clinical importance of oxidative stress injuries and visible benefits of specific antioxidant therapies.

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