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ELECTRON PARAMAGNETIC RESONANCE – A POWERFUL TOOL OF MEDICAL BIOCHEMISTRY IN DISCOVERING MECHANISMS OF DISEASE AND TREATMENT PROSPECTS

ELEKTRONSKA PARAMAGNETNA REZONANCA – MOĆNO ORUĐE MEDICINSKE BIOHEMIJE U OTKRIVANJU MEHANIZAMA OBOLJENJA I MOGUĆIH TRETMANA

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Summary: In pathophysiological conditions related to oxidative stress, the application of selected antioxidants could have beneficial effects on human health. Electron paramagnetic resonance (EPR) spectroscopy is a technique that provides unique insight into the redox biochemistry, due to its ability to: (i) distinguish and quantify different reactive species, such as hydroxyl radical, superoxide, carbon centered radicals, hydrogen atom, nitric oxide, ascorbyl radical, melanin, and others; (ii) evaluate the antioxidative capacity of various compounds, extracts and foods; (iii) provide information on other important parameters of biological systems. A combination of EPR spectroscopy and traditional biochemical methods represents an efficient tool in the studies of disease mechanisms and antioxidative therapy prospects, providing a more complete view into the redox processes in the human organism.

Keywords: EPR, free radicals, oxidative status, oxidative stress, antioxidants

Kratak sadržaj: U patofiziološkim uslovima povezanim sa oksidativnim stresom, primenjivanje određenih antioksidativnih materija može biti od koristi za ljudsko zdravlje. Elektronska paramagnetna rezonantna (EPR) spektroskopija predstavlja tehniku koja pruža jedinstveni uvid u biohemijske redoks procese, zahvaljujući svom kapacitetu da: (i) razlikuje i kvantifikuje različite reaktivne vrste, kao što su hidroksil radikal, superoksid, ugljenični radikali, vodonični atom, azot monoksid, askorbil radikal, melanin i druge; (ii) odredi antioksidativne kapacitete različitih jedinjenje, ekstrakata i namirnica; (iii) pruži informacije o drugim važnim parametrima bioloških sistema. Kombinacija EPR spektroskopije i tradicionalnih biohemijskih metoda predstavlja efikasno oruđe u ispitivanju mehanizama oboljenja i moguće antioksidativne terapije, pružajući kompletniji uvid u redoks procese u ljudskom organizmu

Ključne reči: EPR, slobodni radikali, oksidativni status, oksidativni stres, antioksidanti

Introduction

Electron paramagnetic resonance (EPR) spectroscopy represents a technique which reveals a unique insight into the world of free radicals and antioxidants. Free radicals were first discovered in biological systems more than 50 years ago (1), and soon thereafter they were labeled as »villains«, unwanted but inevitable by-products of our aerobic existence (2). Such perception initiated a 'long-term romance' between medicine and antioxidants, which was fueled by the discovery of enzymes in charge of antioxidative defense (3). Since free radicals are species that contain one or more unpaired electrons, most of them are relatively reactive and capable of oxidizing biomolecules. Due to this property, an uncontrolled generation of radicals is associated with various pathophysiologies such as neurodegenerative diseases (4), malignancy (5), atherosclerosis (6), diabetes (7), sepsis (8), pregnancy complications (9), and also the aging process (10). However, in the 70's and the 80's, the science of free radicals has entered a new era with first reports on the the advantageous effects of reactive species (11, 12). It is clear nowadays, based on a large body of evidence, that living beings have not only adapted to co-existence

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with free radicals, but have developed mechanisms to use them as oxidation/reduction switches for the regulation of gene expression and enzyme activity, in a process known as redox signaling (13–15). It has been documented that redox signaling is involved in the control of vascular tone, oxygen tension, the activity of immune system, and other processes (the list is rapidly growing) (14). In contrast to the previous understanding of the metabolism of antioxidants, it is known now that biosystems have in fact developed refractory response against excessive presence of antioxidants, in order to maintain flexible intracellular redox poise, which is obligatory for normal signaling (15). To conclude, free radicals are not all bad, nor are antioxidants all good, but they are unquestionably very important players in the biochemical processes involved in human physiology and pathophysiology.

In order to get a glimpse of the way in which medical biochemists see the subjects of the present review, I have undertaken a brief systematic search of PubMed (electronic database of U.S. National Library of Medicine) for articles published between 2000 and 2008, that contain a combination of the term 'biochemistry' and a specific phrase or term: 'free radical', 'antioxidant', 'oxidative stress', 'redox signaling', or 'electron paramagnetic resonance' (or its synonym 'electron spin resonance'). Obviously, there is a growing interest in (medical) biochemistry for free radicals, oxidative stress, and particularly for antioxidants (*Figure 1*). On the other hand, the emerging field of redox signaling has not yet attracted deserved attention.

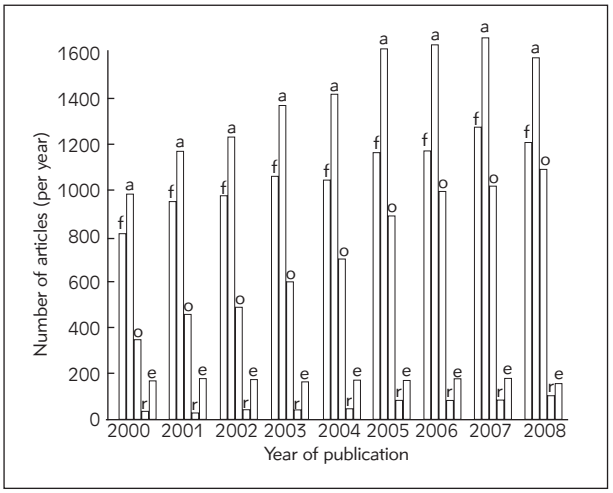

Figure 1 The number of articles (per year of publication) available on PubMed internet database, containing a combination of the term 'biochemistry' and a specific phrase or term: 'free radical' (f); 'antioxidant' (a); 'oxidative stress' (o); 'redox signaling' (r); and 'electron paramagnetic resonance' or 'electron spin resonance' (e). The search was performed using the following limitations: »All fields« and »Specific date range« (from January 1st 200x to December 31st 200x ($0 \leq x \leq 8$)).

The application of EPR in biochemistry is still relatively infrequent, and seems not to keep pace with the rising trend in the free radicals research. This is rather perplexing, as EPR currently represents the only method capable of detecting and discriminating different biological radicals. In line with the above, the aim of this review was to introduce medical biochemists with the principles and capacities of EPR in order to encourage the application of this valuable technique in modern medical biochemistry.

The principles and applications of EPR in medical biochemistry

EPR spectroscopy has been explained extensively in the literature (16–19), so its principles will here be described only briefly. The phenomenon of EPR is based on an intrinsic magnetic moment of the electron that arises from its spin. In most systems, electrons occur in pairs such that the net magnetic moment is zero. However, if an unpaired electron is present, its magnetic moment can suitably interact with a magnetic field. In the field, the unpaired electron spins around the field axis with its magnetic moment either parallel or antiparallel to the field vector, which defines two energy levels (*Figure 2A*). When such a system is exposed to another oscillating magnetic field of resonant frequency, transition between the two levels develops. Since a larger number of unpaired electrons were in the lower energy level, absorption is detected under resonant conditions (similar to optical spectrometry) by the EPR spectrometer (*Figures 2B* and *C*). It is customary to present EPR spectra as the first derivative of the absorption spectrum (*Figure 2C*), because such an approach facilitates spectral analysis. EPR spectra are affected by the nuclei surrounding the unpaired electron (*Figure 2D*) and by some other important phenomena such as molecular motion (20), or interactions with molecular oxygen (21), which enables quantification of viscosity or oxygen concentration using EPR spectroscopy.

The detection of unpaired-electron systems of interest for medical biochemistry implies some technical details, which should be mentioned here. The resonance occurs at a field of about 3000 Gauss, if the frequency is 9–10 GHz (X-band EPR). These frequencies are in the microwave region, which does not affect *in vitro* or *ex vivo* measurements, but for *in vivo* studies lower frequencies and magnetic fields (1-2 GHz and ~400 Gauss; L-band EPR) have to be used, because of the requirements of adequate penetration of radiation and in order to avoid non-resonant absorption (heating) in aqueous biological samples (22). There are two types of EPR spectrometers – CW (continuous wave) and FT (Fourier transform). In the former, more common type of spectrometers, resonant conditions are achieved by changing the magnetic field at a fixed frequency of radiant energy. The principles of FT-EPR are similar to

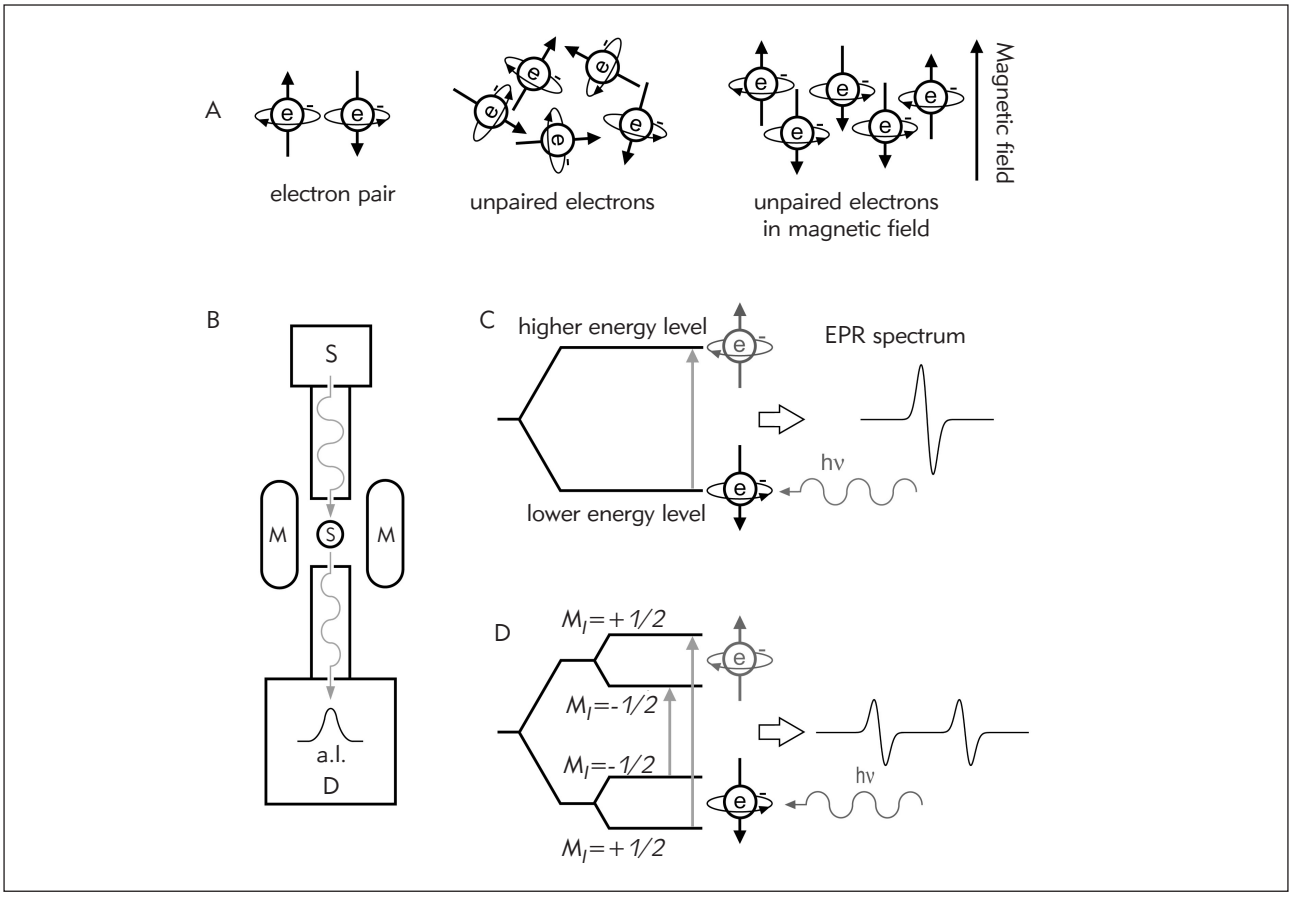

Figure 2 Schematic description of the basic principles of EPR spectroscopy. A) Interaction between electrons and magnetic field; B) scheme of EPR spectrometer (S – source of oscillating magnetic field; M – magnets which generate static magnetic field; s – sample; D – detector; a. l. – absorption line); C) energy-level scheme of unpaired electron showing EPR absorption and corresponding spectrum with one line; D) energy-level scheme of unpaired electron coupled with nucleus with I = 1/2, showing two resonant transitions and corresponding spectrum with two lines.

Table I The characteristics of EPR spectroscopy.

(i)	EPR spectrometer can detect nanomolar concentrations of paramagnetic species.
(ii)	There is a limited number of paramagnetic species in biological systems, so the detection of species of interest is not hampered by a redundant background signals. In addition, this makes the application of various exogenous species (e.g. spin-traps) relatively simple and straightforward.
(iii)	The acquisition of an EPR spectrum usually takes only few minutes.
(iv)	The detection of long lived paramagnetic species intrinsically present in biological systems can be performed without any interference with biochemical processes. For the detection of short lived species and other applications, usually only one selected compound (e.g. spin-trap) has to be introduced into the studied system.
(v)	Measurements usually require less than 100 μL of sample.
(vi)	EPR spectra can be obtained from fluids (plasma, cerebrospinal fluid, extracellular fluid, saliva, etc.), tissues and tissue homogenates, cell cultures, and solid samples (bones, tooth enamel) (25). Measurements can be easily performed at any selected temperature, atmosphere or light regime.
(vii)	EPR spectroscopy enables differentiation between various paramagnetic species with high specificity.
(viii)	Total number of unpaired electrons (and related paramagnetic species) in the sample is proportional to the intensity of corresponding EPR spectrum, which can be established by double integration. The exact concentration is obtained from intensity/concentration calibration curve which can be prepared by using different synthetic paramagnetic compounds.
(ix)	In my experience, the most complete insight into redox processes can be achieved by complementing EPR with biochemical methods, such as the AOS (antioxidative system) enzyme or SH group assays.

those of nuclear magnetic resonance. The spin ensemble placed in a fixed magnetic field is perturbed by a pulse of radiation at or close to the resonant frequency. The detector does not detect the absorption line, but free induction decay of excited electrons, which is then deconvoluted to EPR lines by the Fourier transformation. FT-EPR enables faster spectra acquisition, time-resolved EPR spectroscopy, studies of molecular motion, and improved imaging (19, 23).

The funds that are required for introducing EPR spectroscopy in a laboratory may vary, depending on the desired applications. A modern X-Band EPR spectrometer costs around 200 k€. It enables *in vitro* and *ex vivo* detection of various paramagnetic species (species with unpaired electron(s)) in biochemical systems and biological samples. The maintenance of equipment does not require significant funding and the period of exploitation is over 30 years. Additional 150 k€ are needed for *in vivo* spectroscopy and imaging (including *in vivo* oximetry and NO-metry (19)) instrumentation (L-Band EPR spectrometer). Finally, for a fully equipped EPR laboratory consisting of X-Band EPR, L-Band EPR and FT-EPR spectrometers (24), a total of approximately 900 k€ has to be paid. Presented investments seem reasonable, taking into account the wide range of experiments that can be performed (25), and the characteristics of EPR spectroscopy, which represents a technique that is (i) sensitive, (ii) specialized, (iii) rapid, (iv) principally non-destructive, (v) small sample-requiring, (vi) flexible, both (vii) definitive and (viii) quantitative, and (ix) complementary to biochemical methods (*Table I*).

This paper presents an overview of the applications of EPR spectroscopy in medical biochemistry: direct detection of long lived paramagnetic species, which can be used for oxidative status research (section 3 in this review), for detection of short lived free radicals by the EPR spin-trapping method (section 4), and for the evaluation of antioxidative properties of various compounds by EPR spin-trapping with free radical-generating systems (section 5).

EPR in oxidative status research – the detection of long lived paramagnetic species

The oxidative status of a biosystem represents the relative level of oxidation of biological molecules and structures. Oxidation is involved in cellular signaling, but too much of it may compromise physiological functions. Hence, information about the oxidative status is essential for the understanding of (patho)physiological processes in humans (26). Several biochemical approaches can be used to evaluate the oxiative status, such as ABTS and other spectrophotometric assays (27), measurements of the concentration of R-SH groups (decreased under prooxidative conditions) (28, 29), measurements of the activity of AOS enzymes (Mn- and CuZn-SOD, catalase, GSH peroxidase, and reductase) (27, 30, 31), and fluorescent microscopy with dyes (in)activated by oxidation (32). These techniques are widely used and very informative, but they can be complemented by EPR spectroscopy in order to obtain more specific data about the oxidative status. In most cases, the oxidation of biological molecules leads to 'EPR silent' products. However, some molecules, such as ascorbate, tocopherol, melanin, and others, form radicals (ascorbyl radical, tocopheroxyl radical, semiquinone radical in melanin, etc.) with a long lifetime, allowing their direct detection in human fluids and tissues by EPR spectroscopy (33). The relative levels of these endogenous paramagnetic molecules represent markers of oxidative status, which enables EPR to provide vital data about oxidative conditions inside the biosystems without any interference with intrinsic processes, which is of crucial importance in some studies.

Ascorbyl radical ($^{\bullet}$Asc) has been detected by EPR in a variety of human systems, such as plasma (34–39) and whole blood (40), amnion fluid (30), cerebrospinal fluid (CSF) (39), skin (41, 42), extracellular fluid (43), gastric mucosa (44), and seminal fluid (45). An increase of $^{\bullet}$Asc concentration was reported in several conditions related to prooxidative changes, such as ischemia/reperfusion (40), sepsis (46), brain injuries (47), UV irradiation (42, 43), paraquat poisoning, iron overload, gastritis and gastric cancerogenesis (44), leukemia (39), preeclampsia (48), and amyotrophic lateral sclerosis (ALS) (49, 50). Using EPR, the concentration of $^{\bullet}$Asc can be determined with the lower limit of approximately 5 nmol/L and standard deviation less than 1 nmol/L (36). However, the basal $^{\bullet}$Asc level in tissues and fluids can significantly vary between subjects (35), because it depends on the level of ascorbate in the system (42). This potential drawback has been solved by Galleano and co-workers (51), proposing that the $^{\bullet}$Asc/ascorbate ratio could be used as a marker of oxidative status independent of individual variations. The level of ascorbate can be determined by HPLC (51) or α,α'-bipyridyl method described by Okamura (52). This approach represents a good example of the usefulness of combining EPR with biochemical techniques. It should be noted here that tocopheroxyl radical ($^{\bullet}$TO) is strongly thermodynamically interlinked with ascorbate, via the reaction: $^{\bullet}$TO + Asc→TOH + $^{\bullet}$Asc, so its EPR signal emerges in biological samples only after virtual disappearance of $^{\bullet}$Asc (53). Hence, the presence of $^{\bullet}$TO detected by the means of EPR stands for intense oxidative stress, capable of depleting the antioxidative capacity of ascorbate.

Melanin represents a broad class of biopolymers without a uniform structure, composed of 5,6-dihydroxyindol, 5,6-dihydroxyindol 2-carboxilic acid, and their different oxidized forms (54). In the reactions of melanin with free radicals or catalytic

metals, a stable semiquinone radical and other paramagnetic species detectable by EPR are formed inside the melanin molecule (55). This characteristic makes the intensity of a EPR signal of melanin an excellent marker of oxidative status of melanin-containing systems such as human skin (56) or retina (57, 58). EPR spectroscopy of melanin was shown to be very useful in studying specific pathologies, such as Parkinson's disease (PD) (59, 60), alkaptonuria (61), age-related macular degeneration (AMD) (62, 63), and skin cancer (64). Neuromelanin, which is found in *substantia nigra* and some other brain regions, draws much attention because of its role in PD. It has been documented, using EPR, that neuromelanin shows cytoprotective effects via the sequestration of catalytic metals (59). The production of this pigment is decreased in PD (60), which could lead to oxidative stress and neurodegeneration. Menon and co-workers applied EPR to detect melanin in the urine of alkaptonuria patients (61). They have noted that this pigment provokes cell lysis which is inhibited by catalase. Based on that, the authors have suggested that the alkaptonuria-related tissue degeneration may be due to a prooxidative activity of the metals present in melanin depositions. It has been proposed based on EPR measurements that changes in the retinal melanin may be responsible for AMD, which presents a major cause of late onset blindness in the developed countries (62, 63). Using EPR, the photochemistry of melanin precursors in humans has been studied (64), showing that they could be modified by UV irradiation to potentially mutagenic compounds related to skin cancer development. It should be stressed that there are some other EPR active species of interest for biochemistry, such as amino acid radicals: tyrosyl, tryptophanyl, glycyl, and histidinyl radical, which have been previously employed in studying enzyme activity and protein damage (65–67).

EPR spin-trapping – the detection of short-lived free radicals

High reaction rates and fast relaxation times have conspired to prevent the direct EPR visualization of many physiologically relevant radicals (68). Instead, their involvement in redox processes is usually inferred or confirmed through analysis of products by various biochemical methods (27), or identified by EPR spin-trapping. This EPR technique has a special place in oxidative studies because of its unique ability to identify and quantify the level of any specific short-lived free radical involved in biological processes, including even $^{\bullet}OH$ with the lifetime of $\sim 10^{-9}$s or $^{\bullet}O_2^-$ ($\sim 10^{-6}$s). The basic principles of EPR spin-trapping technique are presented in *Figure 3*. EPR 'silent' compound – spin-trap is introduced into the studied system to 'trap' the reactive intermediate radicals, thus forming stable paramagnetic species – spin-adducts, which are then analyzed by EPR. The

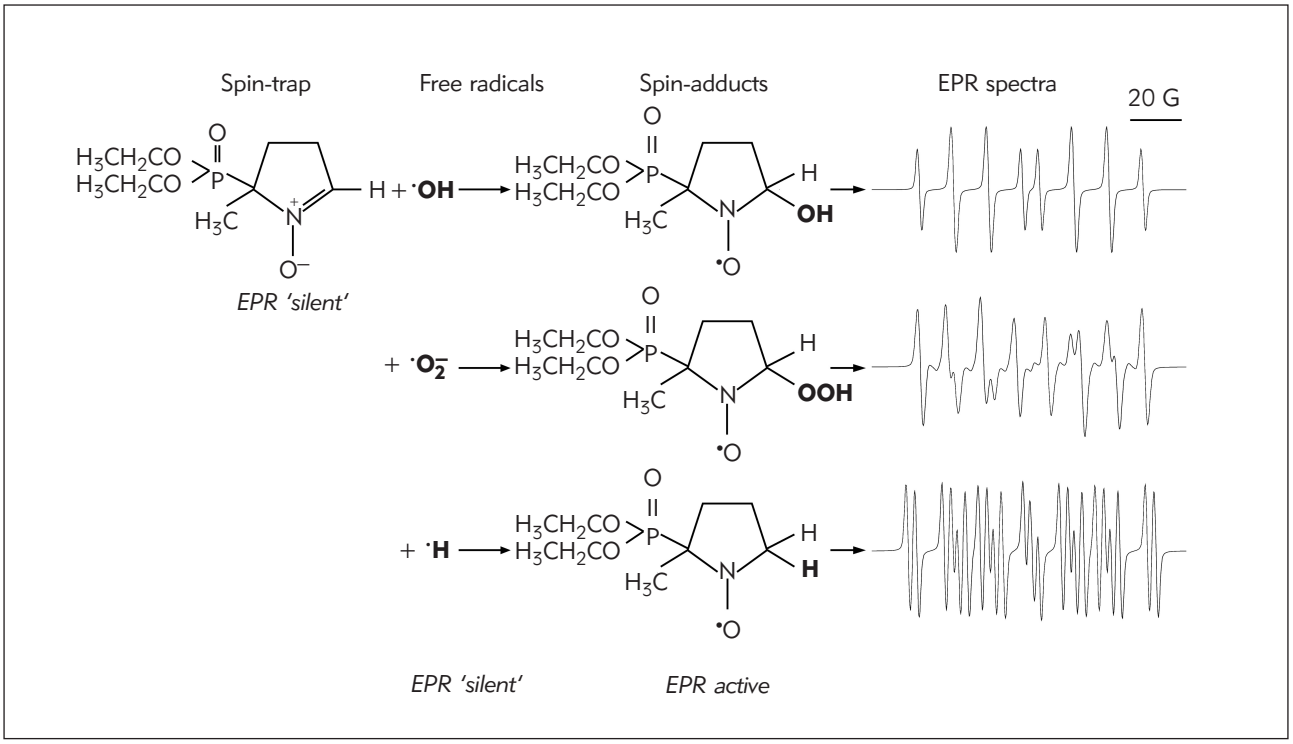

Figure 3 Basic principles of the EPR spin-trapping method, shown on the example of the spin-trap DEPMPO and a set of physiologically most relevant free radicals – hydroxyl radical, superoxide, and hydrogen atom.

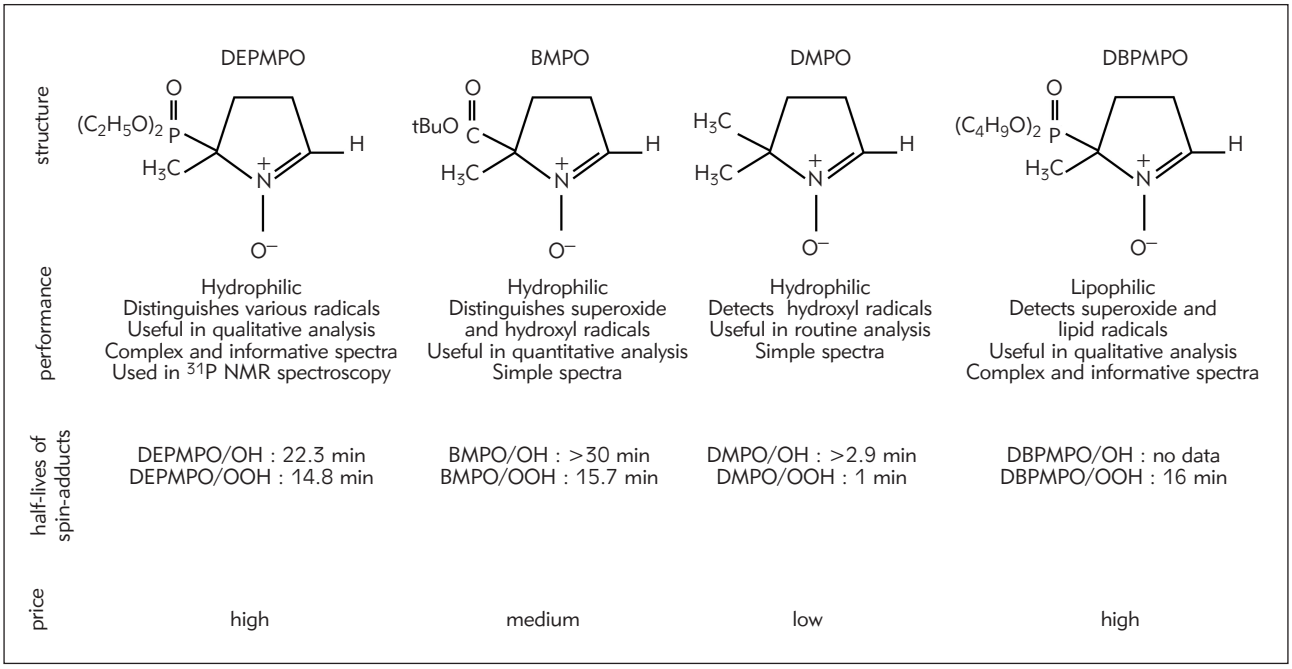

Figure 4 Structures and characteristics of commonly used spin-traps. /OH – spin-adduct of $^{\bullet}OH$; /OOH – spin-adduct of $^{\bullet}O_2^-$ The data were obtained from several papers (70–73, 78). It should be noted that according to the estimation of cell viability (trypan blue exclusion test), DEPMPO, BMPO, DMPO and other spin-traps show no cytotoxicity at standard concentrations (2.5–25 mM) used *in vitro* (79).

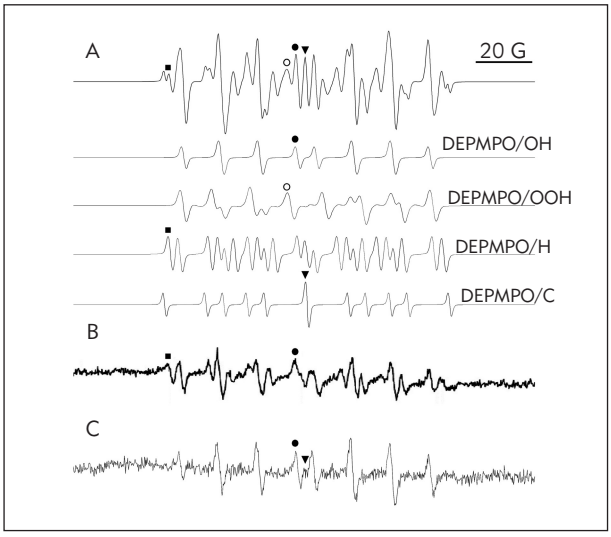

Figure 5 The capability of EPR spin-trapping to distinguish different free radicals. Panel A: Simulated EPR spectrum composed of the signals of four different DEPMPO adducts (dark) in comparison to the simulations of a particular spectrum of each adduct (pale). All spectra are generated by computer simulations. The symbols mark characteristic spectral lines of adducts: filled circle – $^{\bullet}OH$ adduct (DEPMPO/OH); hollow circle – $^{\bullet}O_2^-$ adduct (DEPMPO/OOH); square – $^{\bullet}H$ adduct (DEPMPO/H); downward triangle – $^{\bullet}CH_2OH$ adduct (DEPMPO/C). Panel B: EPR spectrum of DEPMPO spin-adducts obtained from the islets of Langerhans under physiological conditions (70). Panel C: EPR spectrum of DEPMPO spin-adducts obtained from the CSF of ALS patients, supplemented with hydrogen peroxide (49).

structure and EPR signal of a spin-adduct are dependent on the free radical trapped.

Spin-adducts are readily detected by EPR spectroscopy, whereby different species can be distinguished with high sensitivity. For example, the detection of superoxide with EPR and spin-trap DEPMPO is 40 times more sensitive than spectrophotometric analysis with cytochrome *c* (69). The amount of adduct present, which depends on the concentration of a specific radical in the system, is proportional to the intensity of the signal, or more accurately, to the area under the peaks of its EPR spectrum. A variety of spin-traps is now available which can simultaneously trap and tell apart various free radicals, such as $^{\bullet}OH$, $^{\bullet}O_2^-$, $^{\bullet}H$ (70), different carbon-centered radicals ($^{\bullet}CH_3$, $^{\bullet}CH_2OH$, $^{\bullet}CO_2^-$) (71, 72), glutathionyl radical ($^{\bullet}GS$) (72), sulfite anion radical ($^{\bullet}SO_3^-$) (72), lipid radicals ($^{\bullet}LOO$ and $^{\bullet}LO$) (73), and others (74–78). Spin-traps differ in their structure, performance, spin-adduct stability, and prices (*Figure 4*). Instead of using the complicated chemical names of spin-traps, EPR scientists use acronyms such as DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide), BMPO (5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline-*N*-oxide), DMPO (5,5-dimethyl-pyrroline-*N*-oxide), DBPMPO (5-(dibutoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide), etc. (74, 75).

A tremendous number of EPR spin-trapping studies addressing human physiology and pathophysiology have been conducted. This subject has been

reviewed extensively in the literature (74–78), hence only some examples will be listed here. EPR spin-trapping has been employed to study: **(i)** redox metabolism of mitochondria (80, 81), cellular membranes (82, 83), neutrophils (84), platelets (85), endothelial cells (86), spermatozoa (87), CSF (49, 88), the islets of Langerhans (89); **(ii)** redox properties of hemoglobin (90, 91), iron (49, 92), metal-chelates (49), various drugs and toxic compounds (doxorubicin (93), phthalocyanines (94), asbestos (95), paraquat (96), etc.), DNA and proteins (by using immuno-spin-traps (80, 97, 98)); **(iii)** mechanisms of ischemia/reperfusion-provoked tissue damage (86, 99), UV-mediated damage (100), Alzheimer's disease (101), Down's syndrome (102), ALS (49), myotonic dystrophy (103), carcinogenesis (104, 105), aging (106), chronic renal failure (107), pneumoconiosis (108), silicosis (109), ozone toxicity (110), etc. It is important to note that biological systems usually generate more than one free radical. This potential methodological drawback is seemingly insolvable by methods other than EPR spin-trapping, which is capable to simultaneously detect up to three (or even more) different reactive species. The signals of specific spin-adducts can be recognized by their characteristic spectral lines and by performing spectral simulations (*Figure 5A*), which are also used for the purposes of quantification of the spin-adduct (free radical) concentration. *Figures 5B* and *C* illustrate that EPR spin-trapping can be used in studying both the normal functions of biological systems and pathophysiological processes. *Figure 5B* shows that the islets of Langerhans spontaneously generate $^{\bullet}OH$ and $^{\bullet}H$ under physiological conditions. The oxidative status of the islets under such conditions is balanced, since the oxidizing effects of $^{\bullet}OH$ are neutralized by $^{\bullet}H$ representing a reducing agent (70). In contrast, the EPR signal obtained from the CSF of ALS patients (*Figure 5C*) shows that the pathophysiology of ALS may be related to oxidative stress mediated by $^{\bullet}OH$ and carbon-centered radicals (49, 88).

EPR using specific traps is an indispensable tool in studying the metabolism of nitric oxide ($^{\bullet}NO$), which represents a radical species that is crucial in a large number of biomedical settings (14, 88, 92, 111). EPR technique for the measurement of $^{\bullet}NO$ is based on its entrapment to form a stable paramagnetic NO-adduct. The principal approach has been to use metal complexes, such as complexes of ferrous iron and diethyldithiocarbamate (Fe^{2+}-$(DDC)_2$) or methylglucamine-diothiocarbamate (Fe^{2+}-$(MGD)_2$) that trap $^{\bullet}NO$ and form an EPR detectable product (*Figure 6A*) (111–114). The NO-Fe-$(DDC)_2$ adduct is stable and shows a three-line EPR signal (*Figure 6B*), typical of an electron affected by ^{14}N nucleus ($I = 1$). Fe^{2+}-$(DDC)_2$ complex is hydrophobic and readily enters into cells and tissues, while Fe^{2+}-$(MGD)_2$ is more hydrophilic and remains in the extracellular milieu.

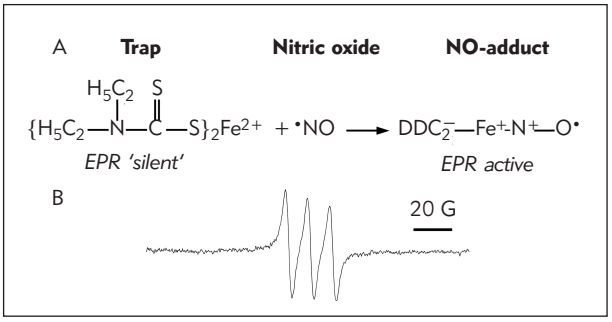

Figure 6 $^{\bullet}NO$ trapping. Panel A: The principles of EPR detection of $^{\bullet}NO$ by using metal complex Fe^{2+}-$(DDC)_2$. In NO-Fe-$(DDC)_2$ adduct, unpaired electron is dislocated over the nitrogen and oxygen atoms. Panel B: Characteristic EPR signal of NO-Fe-$(DDC)_2$.

The presented EPR approach has been used to study $^{\bullet}NO$ metabolism in various tissues and cells *in vitro* and *in vivo*, under both physiological and pathophysiological conditions. For example, EPR was used to study complex interactions between neuronal, endothelial, and inducible $^{\bullet}NO$ synthases (NOS) (115, 116), the neuroprotective role of inducible NOS (117), the mechanism of vasodilatory effects of nitroglycerin (118), *in vivo* production of $^{\bullet}NO$ in the liver (119), ischemic heart (120), brain (121), whole mice (3D $^{\bullet}NO$-imaging) (122), and other systems (123). It should be noted that there is an alternative approach for $^{\bullet}NO$ detection which could be very interesting for biochemists. Nitric oxide can be trapped by hemoglobin (Hb) to form the Hb-NO complex which can be detected by means of both EPR spectroscopy (124) and spectrophotometry (125).

EPR spin-trapping in antioxidant research

According to Barry Halliwell (126), an antioxidant is any substance that, when present at low concentrations in comparison with oxidizable substrates (such as nucleic acids, proteins, lipids), significantly delays or prevents oxidation of those substrates. Antioxidants are the focus of many research groups in the fields of medical biochemistry, pharmacology, and food science. In addition to being interesting in the treatment of various health problems, the isolation or synthesis of novel antioxidants seems to represent a lucrative activity, since they are widely used in food industry (as supplements or food preservatives), pharmacy (as leads for new drugs), and cosmetic industry (as so called 'premature aging' retardants). This could account for the 'explosion' of antioxidant research (see *Figure 1*), in addition to the fact that there is a wide range of relatively simple methods for the evaluation of 'total' and/or 'unspecific' antioxidative capacity of compounds, plant extracts, or foods (reviewed in 27, 126, 127). Methods such as ferric reducing ability of plasma (FRAP), total reactive

antioxidant potential (TRAP), trolox-equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC), and ferrous oxidation-xylenol orange (FOX) are clearly useful for the rapid screening of potential antioxidative effects of various compounds, foods, and extracts, but according to the opinion of the European Food Safety Authority (EFSA) (128), these assays 'do not predict the occurrence of an effect of the food(s)/food constituent(s) on the protection of body cells and molecules from oxidative damage'. Therefore, these methods should be complemented by other techniques, such as EPR, in order to gain more relevant data.

Two crucial questions about the properties of studied antioxidants need to be addressed in order to positively predict beneficial effects on human health: (i) Is the potential antioxidant active against physiologically relevant free radicals? (ii) Is it effective at concentrations achievable *in vivo*? Despite the need for specialized equipment and the challenges faced in its application to biological systems, EPR spectroscopy remains the gold standard by which all other methods of assessing the activity against free radicals are judged. Spin-trapping is the most widely used EPR approach in antioxidant research (74), being capable to measure antioxidative activity (AA) at any selected concentration of the studied compound against any of the physiologically relevant radicals. The basic principle is to use a generating system for a specific radical, and to measure the relative concentration of the radical in the presence and in the absence of the potential antioxidant. The presence of a compound that possesses antioxidative capacity leads to a lower intensity of the spectrum (*Figure 7*).

EPR spin-trapping with the Fenton reaction is the most frequently used approach for studying antioxidative properties. It has been applied to determine the AA against •OH radical of monosaccharides (129), extracts of various plants (130), seeds (131), mushrooms (132, 133), teas (134), and spices (135), fullerenes (136), polysaccharides (137), vitamins (138), chocolate (139), phenolics (140), metal chelators (141), etc. In contrast to the Fenton system which involves iron (or some other catalytic metal), Haber-Weiss-like reaction represents a metal-free •OH-generating system. Hence, the difference between AA (•OH) obtained using the Fenton system and the Haber-Weiss-like system represents a relative measure of the ability of the studied agent to sequester metals. To the author's best knowledge, my co-workers and I were the first to apply this approach to study metal chelating properties (129). We are currently developing an alternative approach for evaluation the ability of compounds to sequester iron. The concept employs spectrophotometric assays to measure the level of hydrogen peroxide following the Fenton reaction in the absence and in the presence of the potential iron chelator. A compound capable of sequestering iron and thus preventing Fenton reaction, should leave some H_2O_2 unreacted, the level of which is proportional to the chelation potential of the studied compound. EPR spin-trapping with the X/XO system has been used in a number of studies to determine AA ($^{\bullet}O_2^-$) of tea extracts (134), fullerenes (136), β-carotene and vitamin E (142), glutathione (143), aminoguanidine (144), lazaroids (145), etc. In addition to AA, antioxidative capacity can be expressed in the form of the EC_{50} value (mg per mL) – an effective

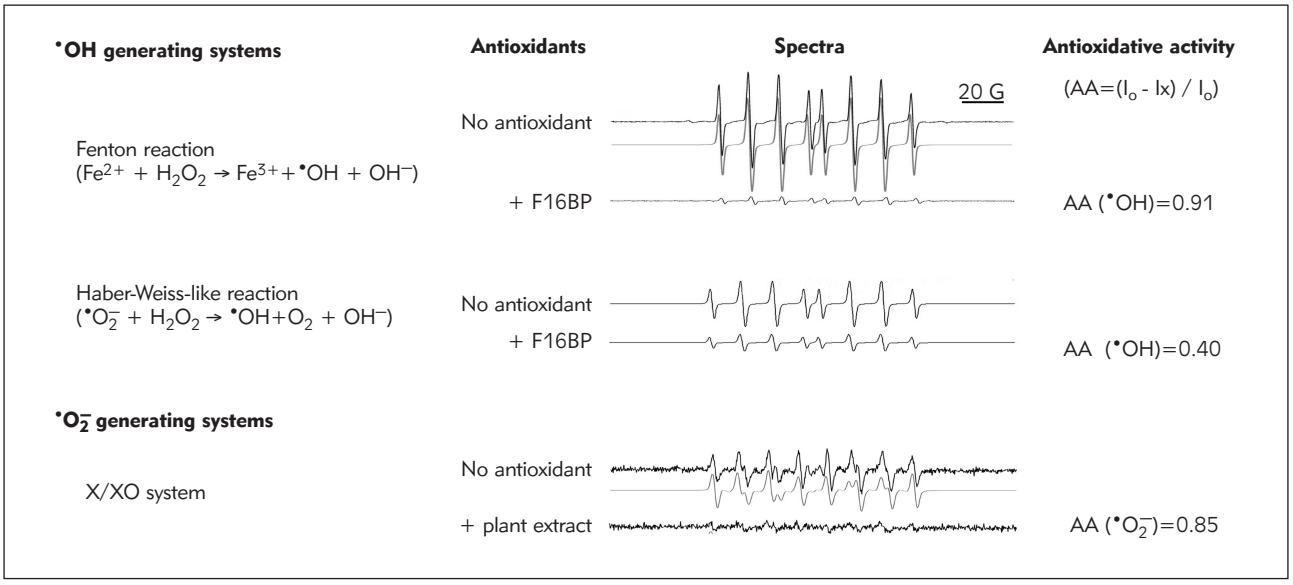

Figure 7 The principles of the application of EPR spin-trapping in antioxidant research as in the examples of F16BP (fructose 1,6-bisphosphate) and the extract of chestnut's catkin (plant extract) (129, 130). Both studies, were performed using physiologically feasible concentrations of potential antioxidants. HX/XO – hypoxanthine/xanthine oxidase system; Io – the intensity of the signal of spin-adduct in the antioxidant-free system; Ix – the intensity of the signal of spin-adduct in the system supplemented with the studied antioxidant. Presented spectra originate from adducts of spin-trap DEPMPO (gray – corresponding spectral simulations).

concentration at which the studied compound or extract shows AA = 0.5 (132, 133). EC_{50} is determined by interpolation from linear regression analysis of several AA values obtained for different concentrations of the compound or extract. This approach is attractive for comparative analysis of data obtained in different studies, but precaution should be taken as EC_{50} depends on the concentration of reactants used in a radical generating system. It should be stressed that there are other radical-generating systems (e.g. for carbon-centered radicals (137) or lipid radicals (142)) that may be used in EPR antioxidant research, and that EPR spin-trapping even enables for the antioxidative activity against several different reactive species to be determined simultaneously in a single generating system/sample (146).

Conclusions

In the era of intense studies of free radicals and antioxidants, electron paramagnetic resonance (EPR) represents a technique of choice for redox research, particularly when biochemical and biological systems are investigated, due to its ability to detect various reactive species, measure antioxidative capacity, and provide other useful information in a sensitive, rapid and relatively simple fashion. The basic principles of EPR technique and three different approaches have been addressed that have not until now received full attention of medical biochemists. The intention was to encourage fellow colleagues interested in redox research to complement the methods used in their laboratories with the EPR techniques outlined in this review in order to further enhance our knowledge in the exciting area of free radical biochemistry.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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