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Research Paper

In vivo evaluation of different alterations of redox status by studying pharmacokinetics of nitroxides using magnetic resonance techniques

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ABSTRACT

Free radicals, particularly reactive oxygen species (ROS), are involved in various pathologies, injuries related to radiation, ischemia-reperfusion or ageing. Unfortunately, it is virtually impossible to directly detect free radicals *in vivo*, but the redox status of the whole organism or particular organ can be studied *in vivo* by using magnetic resonance techniques (EPR and MRI) and paramagnetic stable free radicals – nitroxides. Here we review results obtained *in vivo* following the pharmacokinetics of nitroxides on experimental animals (and a few in humans) under various conditions. The focus was on conditions where the redox status has been altered by induced diseases or harmful agents, clearly demonstrating that various EPR/MRI/nitroxide combinations can reliably detect metabolically induced changes in the redox status of organs. These findings can improve our understanding of oxidative stress and provide a basis for studying the effectiveness of interventions aimed to modulate oxidative stress. Also, we anticipate that the *in vivo* EPR/MRI approach in studying the redox status can play a vital role in the clinical management of various pathologies in the years to come providing the development of adequate equipment and probes.

1. Introduction

Reactive oxygen species (ROS) are generated constantly in living cells. Their overall effect on cells and tissues is determined by the rate of their production and the concentration of low-molecular-weight antioxidants and activity of enzymatic antioxidants which is often described as the redox state or bioreductive capacity of the system (buffer capacity) [1]. Numerous factors can influence the redox state, either by decreasing antioxidant capacity (redox buffer capacity) or increasing the rate of ROS production, both leading to the enhanced oxidative damage of cells and tissues. Oxidative stress has been implicated in a number of pathological conditions such as ischemia, diabetes, neurological disorders, but it can also be a consequence of external factors such as irradiation, intoxication, use of prescribed and other drugs, etc. [2].

It is no surprise, therefore, that numerous studies have been performed while studying ROS in trying to understand the underlying mechanisms with the ultimate goal of preventing or minimizing their deleterious action. Studies of indirect ROS detection range from assessment of the oxidative products (DNA/RNA damage, lipid peroxidation) through measurements of the antioxidant defense system (SOD, glutathione, etc.) to behavioral studies. An ideal technique should be able to directly and non-invasively measure ROS *in vivo*. Spectroscopic techniques that can measure free radicals using probes or directly have been reviewed [3]. Techniques such as fluorescence and luminescence are widely used for studying cells and tissues *in vitro*, but are hardly applicable for *in vivo* studies due to the low penetration depth of used light. Electron paramagnetic resonance (EPR) has advantages since it can, in principle, detect ROS directly and the used electromagnetic waves have sufficient penetration depth for *in vivo* studies, but the situation is not that ideal in real circumstances.

Zavoyski [4] discovered EPR (also called electron spin resonance, ESR) in 1946, almost at the same time when nuclear magnetic resonance (NMR) was discovered. Both techniques were intended as a tool for investigation in solid state physics, but they soon were employed in studying biological/biochemical systems. The early studies were influenced by the low sensitivity of available EPR spectrometers and difficulties in overcoming the problem of non-resonant absorption of microwaves by watery samples. Nevertheless, efforts to study cells and tissues by EPR continued, mostly motivated by the speculations that enzymatic reactions involve the creation of free radicals and that free radicals might be involved in the development of cancer, so that by 1970s EPR became a well-established and respectable technique in the field of biological/biochemical research.

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However, *in vivo* experiments were still beyond reach. The development of the loop-gap resonator in 1982 [5] turned out to be a major breakthrough for *in vivo* EPR. This was soon accompanied by the development of a resonant cavity resonator suitable for whole body experiments on mice [6]. Application of EPR to *in vivo* biological systems essentially started as development of EPR imaging (EPRI) [7,8]. In parallel, extensive work on models and *in vitro* samples has been conducted in establishing basic principles of imaging techniques, contrast enhancement and image reconstruction [9–13]. All this work has been performed by adding external paramagnetic agents, nitroxides (see Section 3), since biological systems do not produce sufficient amounts of radicals to be detected *in vivo*. Next, the first *in vivo* pharmacokinetic experiment has been performed using EPR spectroscopy (EPRS), where injected nitroxides were used to probe redox processes [14]. All these experiments stimulated development of different EPR machines suitable for *in vivo* experiments, and which is equally important, synthesis of new nitroxides that can fulfil specific needs for *in vivo* experiments [15–22]. These articles have been mostly aimed at demonstrating that it is feasible to study the pharmacokinetics of nitroxides, but soon these were followed by studies where the influence of different pathologies on the redox status were investigated (see Section 5). Since the early 90s the field of *in vivo* EPR has grown tremendously in the next two decades to the extent that a complete volume of Biological Magnetic Resonance was needed to cover all the advances and techniques [23]. Much of this work has been stimulated by the discovery that the rate of reduction of nitroxides in cells and tissues is highly dependent on the concentration of oxygen (see e.g. [24,25]).

The realization that one can introduce metabolically responsive and relatively stable paramagnetic free radicals in the body prompted the introduction of another resonance technique (magnetic resonance imaging, MRI) in the field of redox research. At the beginning, nitroxides were studied as potential clinical contrast agents, primarily for tumors [26]. However, relaxation enhancement of nitroxides and corresponding contrast on MR images is around 10 times lower than with a standard MRI contrast agent (Gd-DTPA) per unit of concentration, so little further effort has been put along that line of research. However, with the advent of MRI machines for small animals numerous researches have been recently devoted to studying the redox state under different pathological conditions using nitroxides [27–31].

The main scope of this review is to cover research where the pharmacokinetics of nitroxides has been studied with a goal to investigate redox processes in normal and pathological conditions. The emphasis is on results obtained using EPR techniques, but examples from MRI studies are given when the focus of the study is on the pharmacokinetics of nitroxides and not just imaging using nitroxides as contrast agents. Particular attention is given to how fruitful a combination of EPR and MRI can be in achieving optimal analysis of the investigated subject. Details on EPR imaging alone of the oxidative stress can be found in the recent review [32]. Perhaps the most powerful application of *in vivo* EPRI is measurement of oxygen (EPR oximetry), but this subject will not be covered *per se* in this review. EPR oximetry has been extensively and regularly reviewed [33–35] and oximetry will be considered only when directly connected with the pharmacokinetics of nitroxides. The purpose of this review is not only to summarize results obtained so far but also to point to certain flaws in conducted research and to indicate a possible direction for improvement and development.

2. Technical consideration of magnetic resonance spectroscopy/imaging *in vivo*

Both EPR and NMR are resonant techniques that record spin transitions when a system in the magnetic field is exposed to adequate (resonant) electromagnetic irradiation. Although there are few fundamental differences between the principles of electron and nuclear magnetic resonance, differences in physical properties of the resonant species (unpaired electrons *vs*. nuclei with net spin) lead to profound differences in applications and techniques that are used to record spectra. Perhaps the greatest differences arise because the gyromagnetic ratio of an unpaired electron is ~700 times larger than that of a proton, so the resonance frequency/magnetic field ratio for the electron is 28 GHz/T, *vs*. 42.5 MHz/T for the proton. In principle, EPR is more sensitive than NMR per net spin in the same magnetic field. In practice, however, the situation is different, especially in biological systems. Namely, standard EPR spectrometers operate at much higher frequencies and lower fields than conventional NMR spectrometers. A standard commercial EPR spectrometer operates at 9.5 GHz (X-band) - 0.34 T; while NMR spectrometers operate at frequencies above 500 MHz, i.e. at magnetic fields above 10 T. Clinical MRI machines operate at fields at/or above 1.5 T while small animal imagers typically operate at 7 T or higher. The essential problem of EPR experiments on small animals is the non-resonant absorption of the electromagnetic radiation by the dielectric liquid water in biological systems. At 9.5 GHz this effectively limits the sample size to a thickness of a mouse tail. One could increase the penetration depth of microwaves into larger aqueous samples by increasing microwave power, but that would produce unacceptable heating of the subject (a microwave oven). Hence, animals can be studied only by reducing the operating frequency and corresponding magnetic field, but this results in reduced sensitivity. Studies on small animals have been performed at the S-band (2–3 GHz) with skin depth penetration, L-band (1.2 GHz) or even lower (700 or 280 MHz) for whole body imaging. Another problem is that commercial EPRI machines suitable for *in vivo* applications were not available until recently, hence most of the researchers used (and still are) a homemade apparatus or modifications of commercial ones.

The most important difference between *in vivo* MRI and EPRI is in the species that they detect. Both direct observations and theoretical calculations show that endogenous paramagnetic species such as paramagnetic metal ions and free radicals are present in insufficient concentrations to be detected directly by EPR *in vivo*. This presents an obvious problem since the paramagnetic substance has to be introduced in EPR experiments. On the other hand, this has some beneficial aspects, since the only paramagnetic species that will be observed *in vivo* will be those introduced by the experimenter. Also, the behavior of introduced nitroxides can be related to metabolism. MRI sees water protons which are abundant, enabling excellent images (water is 55 M and nitroxides are usually injected at doses around 1 mmol/kg of body weight). Nitroxides are seen indirectly through their effect on the relaxation of water protons. EPRI does not provide images of anatomy; it just shows the distribution of injected nitroxide within the body and does not have very good spatial resolution. Conversely, MRI has excellent spatial resolution and provides detailed anatomical information, but gives little information on the paramagnetic species involved. Both techniques have their advantages and drawbacks in *in vivo* ROS detection/imaging, thus the sensible simultaneous use of both is a way to employ the optimal potential of these techniques.

For example, it is possible to use both techniques in imaging modality and overlay EPRI providing redox information on top of MRI providing anatomical information [36,37]. There were also constructions of dual EPR/MR imaging machines where both types of information can be obtained without moving the animal [30,38,39]. Probably the best way to fuse EPR and MRI into a single machine is to use dynamic nuclear polarization (DNP or Overhauser effect, OMRI) which uses a unique method for detection of