Recent Progress in *In Vivo* ESR Spectroscopy

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*In vivo* ESR/Free radicals/Redox/Partial oxygen pressure/Oxidative stress

The generation of free radicals and redox status is related to various diseases and injuries that are related to radiation, aging, ischemia-reperfusion, and other oxidative factors. *In vivo* electron spin resonance (ESR) spectroscopy is noninvasive and detects durable free radicals in live animals. ESR spectrometers for *in vivo* measurements operate at a lower frequency (~3.5 GHz, ~1 GHz, 700 MHz, and ~300 MHz) than usual (9-10 GHz). Several types of resonators have been designed to minimize the dielectric loss of electromagnetic waves caused by water in animal bodies. *In vivo* ESR spectroscopy and its imaging have been used to analyze radical generation, redox status, partial pressure of oxygen and other conditions in various disease and injury models related to oxidative stress with probes, such as nitroxyl radicals. Through these applications, the clarification of the mechanisms related to oxidative diseases (injuries) and the accumulation of basic data for radiological cancer therapy are now ongoing. *In vivo* ESR measurement is performed in about 10 laboratories worldwide, including ours. To introduce *in vivo* ESR spectroscopy to life scientists, this article reviews the recent progress of *in vivo* ESR spectroscopy in instrumentation and its application to the life sciences.

**INTRODUCTION**

Ever since the suggestion of a relationship between reactive oxygen species and disease (or aging), the measurement of biological free radicals and redox states has been a popular research topic. Electron spin resonance (ESR) spectroscopy is the most reliable technique for this purpose because it measures only paramagnetic species having unpaired electrons, such as free radicals and transition metal complexes. This technique has been used *in vitro* to measure oxygen radicals such as hydroxyl radical (·OH) and superoxide anion radical (O₂⁻) in combination with the spin-trapping technique¹. The measurement of ESR is nondestructive and is unaffected by the turbidity of the sample, so people are interested in using ESR for the *in vivo* measurement of biological radicals. However, there are difficulties with this. First, steady concentrations of biological radicals are too low to detect directly with ESR spectroscopy during their very short half-life. Second, water in the body of the animal causes dielectric loss of the electromagnetic waves used for ESR measurement. In this article, an outline of *in vivo* ESR is given first, and recent applications of this technique are then summarized.

**ESR SPECTROSCOPY AND DEVELOPMENT OF THE IN VIVO ESR SPECTROMETER**

ESR spectroscopy, like nuclear magnetic resonance (NMR) spectroscopy, is a kind of magnetic resonance spectroscopy. The spin of an electron produces a magnetic moment. Electromagnetic wave radiation of the appropriate frequency under a given external magnetic field causes the excitation of unpaired electrons from the lower energy level to the higher level by the interaction of the magnetic moment of electron spin with the magnetic component of the electromagnetic wave (magnetic resonance). ESR spectroscopy is usually done by the use of continuous wave (CW) methods, in which a continuous radiation of electromagnetic waves is applied to the sample and magnetic resonance is detected by slowly sweeping the external magnetic field.

The ESR spectrometer detects the absorption of the energy of the electromagnetic wave at the resonance frequency. The ESR of *in vitro* samples is conventionally measured with X-band electromagnetic wave (9-10 GHz). Because the dielectric loss of electromagnetic wave is high at this frequency, a small volume (less than 100 µL) of aqueous sample is taken in a thin quartz cell for the measurement. The penetration of the electromagnetic wave depends on the dielectric constant and conductivity of tissues and the frequency of the electromagnetic wave applied. Halpern and Bowman² plotted the penetration depth of electromagnetic wave in a cylindrical geometry as a function of electromagnetic wave frequency.

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In this plot, the radius of the muscle equivalent cylinder (for which the magnitude of magnetic field of electromagnetic wave along the center axis has fallen to \(1/e\) of its surface value) is only ~1 mm at X-band. However, the radius increases with a decrease in the frequency; approximately 16 mm at L-band (~1 GHz) and approximately 55 mm at 250 MHz. Thus the use of lower frequencies is essential for the in vivo application of ESR, especially whole-body measurement of small animals, at the expense of sensitivity. The first noninvasive study was reported in 1983 by Lukiewicz and Lukiewicz\(^3\). They injected durable nitroxyl radical to mouse, and ESR measurement was carried out at L-band in whole body or in melanoma implanted into the tail of the mouse. Subsequently, Berliner et al.\(^4\) imaged the distribution of nitroxyl radical in the melanoma. After these reports, several research groups actively developed the in vivo ESR spectrometer and began the in vivo measurement with live animals. S-band (~3.5 GHz) has been used for the measurement of the tail\(^5-7\). L-band (~1 GHz) has been widely used to measure durable nitroxyl radical in the mouse head, chest, and abdomen\(^8-13\). For whole-body measurement of rats, lower frequencies (280-700 MHz) should be used\(^14-18\). The term radio frequency (RF) in this article indicates the region of electromagnetic wave used for in vivo ESR spectroscopy.

The ESR spectrum recorded under a linear field gradient includes one-dimensional spatial information. To extract the spatial information, the spectrum is deconvoluted with a spectrum recorded under no gradient. The distribution of radicals can typically be imaged by the use of reconstruction methods such as back projection with the one-dimensional radical distributions obtained under field gradients in various directions (refer to reviews\(^19, 20\)). First, a one-dimensional image at L-band was reported by Berliner and Fujii with a celery sample soaked in an aqueous solution of nitroxyl radical\(^21\). Figure 1 shows an ESR imaging system in our laboratory.

**RESONATORS FOR IN VIVO ESR**

Cylindrical and rectangular cavity resonators with specific wave guide modes are commonly used for the X-band ESR spectrometer. In these resonators, a small volume sample receives a high density RF magnetic component to obtain high sensitivity. To apply ESR to in vivo measurements, however, the geometrical shape and dimension of the resonator should be suitable for animal measurements. The resonators for in vivo ESR spectroscopy are classified into two types.

(i) Volume-coil-type resonators

An animal (or a part of an animal) is placed in the resonator, and radicals distributed in the body are measured. This resonator is suitable for whole body measurement of radicals and imaging of the distribution of the radicals.

(ii) Surface-coil-type resonators

The resonator is put on the surface of the animal body (or an organ) like a stethoscope, and radicals present near the resonator are measured rather selectively. This resonator is suitable for the topical measurement of radicals.

The sensitivity of ESR is proportional to the quality factor (Q) of the resonator and the filling factor (\(\eta\)), which is the RF magnetic field-squared weighted fraction of the resonator volume occupied by the sample\(^22\).

The dielectric loss of electromagnetic waves is caused by interaction between the RF electric component and the aqueous sample. Thus the separation of the RF electric field from the RF magnetic field is important for an accurate ESR measurement. The following resonators were designed for this purpose.

**Loop-gap resonator**

This resonator is similar in design to magnetrons and heavy ion particle accelerators and was first used for ESR by
Froncisz and Hyde\textsuperscript{23}. It consists of a loop divided into sections by one or more gaps (Fig. 2). An inductive coupler is used to match the RF line to the resonator by mechanically changing the separation of the coupling loop and the resonator loop. The loop and gap parts of this resonator operate as inductive coil and capacitors, respectively. The capacitive and inductive elements are separated in space, and the RF magnetic field inside the loop is fairly separated from the RF electric field around the gaps. The advantage of this design is that a resonator with a large diameter can be built by increasing the number of gaps. A large diameter resonator needs a shield case to block electromagnetic wave radiation.

The leakage of the RF electric component into the sample space sometimes reduces the Q value and shifts the resonance frequency when the resonator contains a sample with large dielectric constant. Ono \textit{et al.}\textsuperscript{24} shielded gaps on the inside of the resonator loop to prevent leakage of the RF electric component into the sample space (a bridged loop-gap resonator). This approach is valid for the measurement of a relatively large animal. Recently, Ono \textit{et al.}\textsuperscript{25} evaluated the relationship between magnetic field homogeneity in the sample space and the size of the electric shielding plate. The optimum bridge angle to obtain homogeneous sensitivity in the sample space of a loop-gap resonator (70 mm in diameter, 2 gaps) with a resonance frequency of 302 MHz was 80–100°. Zweier and Kuppusamy\textsuperscript{26} separately reported that recessing the gaps of the resonator (1.1 GHz) with semicylindrical holes decreased the fringe of the RF electric field in the sample space, resulting in a decrease in dielectric loss.

A loop-gap resonator was also used as a surface-coil-type resonator using leakage of an RF magnetic component at one end of the loop, not only as a volume-coil-type resonator\textsuperscript{27}.

\textbf{Re-entrant resonator}

The re-entrant resonator was originally developed by Sotgiu \textit{et al.}\textsuperscript{28,29} and is typically composed of two circular channels surrounding central gaps (Fig. 3). An animal is inserted into the center arm, and an inductive coupler loop is inserted into one of the two lateral arms. Matching between the resonator and the RF line is performed by rotating the coupling loop. There is an RF electric field in the gap (re-entrant), and an RF magnetic field distributes in the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.jpg}
\caption{Loop-gap resonator with two-gaps. Matching of the RF line to the resonator is performed by mechanically changing the distance between the coupling loop and the resonator loop.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.jpg}
\caption{Re-entrant resonator. Matching of the RF line to the resonator is performed by rotating the coupling loop.}
\end{figure}
channels to form a lumped circuit. The size of the resonator should be smaller than a quarter of the resonant electromagnetic wavelength.

**Flat-loop coil resonator**

This resonator is composed of a parallel wire transmission line with a short-circuited loop at one end. RF electric and magnetic components distribute to the parallel line and the loop portion, respectively. In the original design, the resonator was mechanically matched to the RF line with a stub tuner. Recent improvements have made the matching easy by the use of varactor diodes or an electrical tuning circuit. The latter is used with an automatic matching control (AMC), which is useful for reducing noise generated by animal movement. The sensitivity map for the flat-loop coil resonator is characteristic. Sensitivity is highest around the plane of the loop, and it decreases exponentially with increasing a distance between the plane and the sample. For this reason, this resonator has been used as a surface-coil type resonator as well.

**Parallel coil resonator**

Devasahayam et al. designed a parallel coil resonator for 300 MHz ESR, which is equivalent to stacked flat-loop coils. It is used as a volume-coil-type resonator because sensitivity is homogeneous in the sample space.

**APPLICATION OF IN VIVO ESR SPECTROSCOPY**

Although the subjects of ESR spectroscopy are limited to compounds with an unpaired electron, the applications of in vivo ESR cover a broad range. Many investigators have applied in vivo ESR spectroscopy and its imaging to the physiological, medical, and pharmaceutical fields. pH in mouse stomach has been monitored with a pH-sensitive nitroxyl probe administered orally. The imaging of cross sections is also possible if the sample is inserted into the loop.

**Redox status**

The pharmacokinetics of compounds carrying nitroxyl radical (nitroxyls) has been studied in animals and plants since the development of in vivo ESR spectrometers. The kinetics of ESR signal decay of pyrrolidine nitroxyls (five-membered rings) and piperidine nitroxyls (six-membered rings) with various substituents was studied in mouse stomach. In vitro studies demonstrated that the reduction of nitroxyl radical was reversible in biological samples, such as liver.
Nitroxyl radical loses its paramagnetism not only by reduction, but also by reaction with OH and carbon-centered radicals at a nearly diffusion-controlled rate. Signal reduction by peroxyl radicals was also reported. Superoxide anion radical caused signal reduction in the presence of reductants such as sulfhydryl compounds and NAD(P)H ($k=10^{7.5}$ $M^{-1}s^{-1}$). As mentioned in the introduction, a direct detection of biological radicals with in vivo ESR spectroscopy is difficult. The reactivity of nitroxyls with some radicals suggests the feasibility of an indirect measurement of radical generation via the reduction of ESR signal of nitroxyls monitored by in vivo ESR spectroscopy. We examined the signal reduction of pyrrolidine and piperidine nitroxyls with various substrituents by in vitro reaction with OH, O$_2^-$, plus reductants. The signal reduction of piperidine nitroxyls varied depending on the substituents in both reaction systems, whereas that of pyrrolidine nitroxyls did not. Pyrrolidine nitroxyls lost paramagnetism by reaction with OH at a near diffusion-controlled rate, but they were generally resistant to signal reduction by O$_2^-$ plus reductants. Furthermore, hydrogen peroxide and singlet oxygen caused no signal reduction in either pyrrolidine or piperidine nitroxyls. These observations suggest that pyrrolidine nitroxyls are relatively specific to OH comparing with piperidine nitroxyls.

Miura et al. measured the rate of signal decay of carbamoyl-PROXYL intravenously injected in mice with an L-band ESR spectrometer equipped with a loop-gap resonator and observed an increase in the rate under hyperoxia. They speculated that nitroxyl reacted with reactive oxygen species, resulting in a loss of paramagnetism, because the enhanced signal decay was suppressed by the preadministration of antioxidants such as glutathione, Trolox (a water-soluble analogue of vitamin E), and uric acid. The irradiation of mice with X-rays (up to 15 Gy) also increased the decay rate of the carbamoyl-PROXYL signal 1 h after irradiation, and the increase was suppressed by the administration of cysteamine before the irradiation. The increase in the signal decay correlated with lipid peroxidation in the liver. The suppression effects on the increased signal decay caused by X-ray irradiation were screened with several radioprotective agents.

The subcutaneous administration of ferric citrate increased the decay rate of carbamoyl-PROXYL signal in mice. This increase was suppressed by the administration of a chain-breaking antioxidant, Trolox. The administration of desferrioxamine, an iron-chelator, also suppressed the increased signal decay, depending on the time of administration with respect to iron loading. In that case there was a good correlation between the ESR signal decay and iron content and lipid peroxidation in the liver. Carbamoyl-PROXYL inhibited the lipid peroxidation of liver homogenates caused by iron citrate. These observations indicate the...
relationship between the increase in the signal decay rate and the free radical reactions.

An increase in the decay rate of carbamoyl-PROXYL was also observed in rats with streptozotocin-induced diabetes by the use of a 300 MHz ESR spectrometer equipped with a loop-gap resonator\(^{48}\). It was suppressed by the administration of \(\text{O}_2^-\) inhibitors, such as superoxide dismutase (SOD), 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron), allopurinol, and oxipurinol, indicating the increased generation of \(\text{O}_2^-\) in the mice\(^{73}\). This was supported by an increased xanthine oxidase level in the plasma of the diabetic mice.

A membrane-impermeable nitroxy probe may useful to measure radical generation in a localized area. Han et al.\(^{74}\) injected a small volume of physiological solution of nitroxy probe carrying a positively charged group, trimethylammonium-TEMPO, into mouse lung via the trachea and measured chest position with an L-band ESR spectrometer equipped with a loop-gap resonator. The ESR signal decreased with time at a rate much lower than that for carbamoyl-PROXYL injected intravenously. The reduction of nitroxy should occur in the lung parenchymal cells\(^{66,77}\). The slow decay rate is probably due to the restricted diffusion of the probe into the cells for its charged group. The analyses of in vivo radical reactions with structurally different nitroxy probes were recently reviewed\(^{26}\).

The oxidation of hydroxylamine is an another potential index for radical reactions, because a hydroxylamine is easily oxidized by biological oxidants including reactive oxygen species to form nitroxyl radical. The apparent rate constants for the reaction of cyclic hydroxylamines with \(\text{O}_2^-\) and peroxynitrite are \(1.7 \times 10^3 - 6.7 \times 10^3 \text{ M}^{-1}\text{s}^{-1}\) and \(4.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}\), respectively\(^{76,77}\). A pioneer study performed in situ with hydroxylamine suggested the possibility of the detection of reactive oxygen species generated under oxidative stress such as the ischemia-reperfusion of organs\(^{78}\).

We injected ACP into mice intravenously or intraperitoneally and examined its pharmacokinetics\(^{81}\). ACP injected intravenously was distributed quickly over most organs. A rapid hydrolysis of ACP occurred in the liver and kidney. The distribution of corresponding hydroxylamine was nearly homogeneous over most tissues 10 min after the injection of ACP regardless of the injection route. The level of hydroxylamine in the tissues was kept more than 30 min after the injection. These observations suggest that ACP is suitable to delineate the area where reactive oxygen species are produced in various disease models related to oxidative stress, using the in vivo ESR imaging technique.

Thus both nitroxy radical and hydroxylamine are potential probes for the evaluation of radical reactions. However, it should be noted that the reactions of these probes are not specific to reactive oxygen species. The alterations of various reductase activities and antioxidant levels modify the redox state of these probes to affect the ESR signal intensity. Furthermore, if the pharmacokinetics of probes is changed in the disease model, it should also affect their signal intensity. Therefore the effects of administering appropriate radical scavengers on the disappearance or appearance of the ESR signal should be examined to assess the radical reactions by the use of the in vivo ESR technique with these probes.

**Spin adducts of biological radicals**

The direct detection of low concentrations of short-lived biological radicals is very difficult. A few research groups have reported the in vivo detection of spin adducts formed as a result of spin trapping biological radicals in vivo. The spin adducts of a conventionally used spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), are unstable in biological systems. Although the adducts of \(\alpha\)-(4-pyridyl 1-oxide)-\(\gamma\)-tert-butyl nitronitrone (POBN) with carbon-centered radicals are stabler in biological systems, an adduct of this spin trap with OH is easily decomposed by itself. The \(\cdot\text{OH}\) reacts with ethanol and dimethyl sulfoxide at a near diffusion-controlled rate to generate 1-hydroxyethyl radical (\(\cdot\text{CH(OH)}\text{CH}_3\)) and methyl radical, respectively. These reactions appear specific to \(\cdot\text{OH}\). Halpern et al.\(^{82}\) injected POBN and ethanol into a tumor implanted in the leg of mouse, irradiated it with \(\gamma\)-rays at 3,000 Gy, and subjected it to 260 MHz ESR measurement. They observed the signal of the \(\cdot\text{CH(OH)}\text{CH}_3\) adduct of POBN in the tumor. This was the first report about in vivo detection of reactive oxygen species by the use of in vivo ESR spectroscopy, though the radiation dose they used was...
preposterous.

A recently developed spin trap, 5-diothoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO), has some advantages in the stability of its spin adducts. Liu et al.83) observed that the sulfur trioxide anion radical (SO\textsuperscript{3–}) adduct of DEPMPO was much more stable than that of DMPO against reduction caused by ascorbic acid in vitro. They injected DEPMPO, sodium sulfite, and sodium dichromate into mice. The reaction of sulfite with dichromate produces SO\textsuperscript{3–}84). They used this reaction as a model system for radical generation. When the mice set in a loop-gap resonator were measured with an L-band ESR spectrometer, a distinct signal for the SO\textsuperscript{3–} adduct of DEPMPO was recorded. Subsequently, an in vivo detection of the .OH spin adduct was performed by the use of a similar method85). The .OH generated by the reaction of FeNH\textsubscript{4}(SO\textsubscript{4})\textsubscript{2} and 5-aminolevulinic acid in vivo, correlates with a concentration of molecular oxygen. Thus they concluded that the .OH adduct was, for the first time, detected in vivo.

On the other hand, there is no report to date about the sulfur trioxide anion radical (SO\textsuperscript{3–}) adduct of DEPMPO, has some advantages in the stability of its spin adducts. Liu et al.83) observed that the sulfur trioxide anion radical (SO\textsuperscript{3–}) adduct of DEPMPO was much more stable than that of DMPO against reduction caused by ascorbic acid in vitro. They injected DEPMPO, sodium sulfite, and sodium dichromate into mice. The reaction of sulfite with dichromate produces SO\textsuperscript{3–}84). They used this reaction as a model system for radical generation. When the mice set in a loop-gap resonator were measured with an L-band ESR spectrometer, a distinct signal for the SO\textsuperscript{3–} adduct of DEPMPO was recorded. Subsequently, an in vivo detection of the .OH spin adduct was performed by the use of a similar method85). The .OH generated by the reaction of FeNH\textsubscript{4}(SO\textsubscript{4})\textsubscript{2} and 5-aminolevulinic acid in vivo, correlates with a concentration of molecular oxygen.

Nitric oxide (NO) has also been subjected to in vivo ESR measurement. The reaction is summarized in Scheme 3. The formation of a paramagnetic mononitrosyl-iron complex as a result of trapping NO with diethylthiodicarbamate (DETC) in vivo was first reported by Vanin’s group86). Lai et al.6,7) developed water-soluble derivatives of dithiocarbamate, N-methyl-D-glucamine dithiocarbamate (MGD), and measured the ESR signal of the MGD-Fe\textsuperscript{2+}-NO complex in the tails of NO donor-injected mice and lipopolysaccharide (LPS)-induced septic-shock mice by the use of an S-band (3.5 GHz) ESR spectrometer equipped with a loop-gap resonator. The signal of the mononitrosyl-iron complex formed in LPS-treated mice was also detected in the abdominal region of DETC-injected mice by the use of an L-band ESR spectrometer equipped with a loop-gap resonator87). Yoshi-mura et al.88) developed dithiocarbamate derivatives with more water solubility, N-(dithiocarboxy)sarcosine (DTCS). They injected a DTCS-Fe\textsuperscript{3+} complex into LPS-treated mice and observed the distinct triple-line signal of the mononitrosyl iron complex in the abdominal region with a 700 MHz ESR spectrometer. Both ESR imaging and in vivo measurement of resected tissue indicated the presence of a high concentrations of the DTCS-Fe\textsuperscript{3+}-NO complex in the liver. Some dithiocarbamate derivatives of amino acids were synthesized, and their NO-trapping efficiency was examined in LPS-treated mice89). The mononitrosyl-iron complex was detected in the liver and blood for serine-derivatives, but the complex was detected only in the blood for proline-derivatives.

Partial pressure of oxygen

Molecular oxygen in its ground state is biradical. Therefore the interaction of stable paramagnetic compounds with molecular oxygen shortens the ESR relaxation times of the compounds, resulting in an increase in the peak-to-peak line width of their ESR spectra. The increase in the line width correlates with a concentration of molecular oxygen. Thus this phenomenon can be used for the measurement of partial pressure of oxygen, pO\textsubscript{2} (ESR oximetry). Available paramagnetic probes fall into two types, water-insoluble particle type and water-soluble type. Lithium phthalocyanine\textsuperscript{91}, fusinite\textsuperscript{92}, synthetic chars\textsuperscript{93}, and India ink\textsuperscript{94} are the former type, and nitroxyls\textsuperscript{95–97} and triaryl methyl (trityl) radicals\textsuperscript{98,99} are the latter. In some studies, nitroxyls were encapsulated into oxygen-permeable capillaries\textsuperscript{95} and liposomes\textsuperscript{96,100} to prevent the reduction and diffusion of the probes. Water-soluble probes were used for ESR imaging of the oxygen map, and water-insoluble probes and encapsulated nitroxyls were suitable for the topical measurement of pO\textsubscript{2}.

Goda et al. implanted India ink in mouse mammary adenocarcinomas (MTG-B) and RIF-1 tumors in mouse legs and irradiated the tumors with X-rays\textsuperscript{101}. As measured with an L-band ESR spectrometer, pO\textsubscript{2} levels in the MTG-B tumors were lower than those in RIF-1 tumors before irradiation. A temporal decrease in pO\textsubscript{2} was observed in both tumors after irradiation. The administration of insulin increased pO\textsubscript{2}, measured with a charcoal probe, in transplantable liver tumors and syngeneic FSAII tumors in mouse legs, which correlated with the increase in the regrowth
delay caused by X-ray irradiation\textsuperscript{102}. The effect of a chemotherapeutic drug, vinblastine, on pO\textsubscript{2} was also examined in another tumor model\textsuperscript{103}. In RIF-1 tumors, pO\textsubscript{2}, measured with lithium phthalocyanine, increased by carbogen-breathing\textsuperscript{104).

Velan \textit{et al}\textsuperscript{97} obtained pO\textsubscript{2} images of mouse tails by the use of a spectral-spatial technique that makes it possible to separate spectral and spatial information. Spectral-spatial imaging with CW-ESR spectroscopy requires a very high field gradient, and consequently the diameter of the sample is limited.

Recent progress in electrical technology has enabled pulsed-radiofrequency ESR imaging of stable radicals with short relaxation times\textsuperscript{105,106}. The interaction of the radical with molecular oxygen shortens the overall time of free induction decay of the excited radical with a short RF pulse. With the use of this time difference, which depends on oxygen concentration, a trial study was performed by Subramanian \textit{et al}\textsuperscript{97} to obtain ESR images of oxygen maps of scc tumors in mouse leg without a large field gradient.

**CONCLUSION**

Although the direct measurement of biological radicals is presently difficult, several studies have been performed with \textit{in vivo} ESR spectroscopy. This may indicate the necessity of the \textit{in vivo} evaluation of radical generation and redox status. The knowledge of the generation routes of radicals is important to clarify mechanisms related to diseases and injuries caused by radiation, ischemia-reperfusion, and other oxidative factors. The pO\textsubscript{2} level should closely relate to the generation of radicals and the progression of their reactions. The sensitivity of the \textit{in vivo} ESR instruments and the specificity and stability of probes are, at present, insufficient. Further improvements of the instruments and probes may give us much more information about the mechanisms for the diseases and injuries from the viewpoints of radical generation and redox status.

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