Clinical Electron Paramagnetic Resonance (EPR) Oximetry Using India Ink

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Abstract Electron paramagnetic resonance (EPR) oximetry can be used to provide direct absolute measurements of pO_2 in living tissue using India ink as an O_2 reporter. In vivo measurements are made using low frequency (1.2 GHz) EPR spectroscopy and surface loop resonators, which enable measurements to be made at superficial sites through a non-invasive (after placing the ink in the tissues) and repeatable measurement procedure. Ongoing EPR oximetry studies in human subjects include measurement of subcutaneous pO_2 in the feet of healthy volunteers to develop procedures that could be used in the treatment of peripheral vascular disease and oximetry in tumors during courses of radiation and chemotherapy, to follow pO_2 so oxygen-dependent therapies can be optimized. In each case, we aim to provide quantitative measurements of tissue pO_2 which will aid physicians in the characterization of disease status and the effects of therapeutic measures, so that treatments can be applied with optimal effectiveness by taking into account the oxygen-dependent aspects of the therapy. The overall goal is to enhance clinical outcomes. Oximetry measurements of subcutaneous tissue on dorsal and plantar foot surfaces have been made in 9 volunteers, with measurements ongoing for each and the longest set of measurements carried out successfully over the last 5 years. Tumor oximetry measurements have been performed in tumor tissues of 10 patients during courses of radiation and chemotherapy. Tumor types include melanoma, basal cell, soft tissue sarcoma, and lymphoma, and measurement sites have ranged from the feet to the scalp. These studies demonstrate the feasibility of EPR oximetry in a clinical setting and the potential for more widespread use in the treatment of these and other oxygen-dependent diseases.

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1 Introduction

In vivo electron paramagnetic resonance (EPR) is a magnetic resonance technique that can be used for functional spectroscopy or imaging of living systems, reporting such parameters as partial pressure of oxygen, free radical concentration, microviscosity, and redox status [1]. EPR measures the absorption of energy, at radio- or microwave frequencies, by unpaired electrons present within the sample and the recorded absorption spectrum contains information about the magnetic environment. As most free radicals present in biological systems are short-lived and present in low concentrations, for most in vivo EPR measurements an exogenous molecular probe is either injected or implanted and acts as a reporter of the physiologic parameter of interest. Many such probes exist, including water soluble molecules [2] and particulate materials [3], with specific sensitivities to particular biologic parameters.

The ability of in vivo EPR techniques to provide repeated non-invasive measurements of tissue oxygenation is of particular interest due to the critical importance of O_2 in normal physiology and disease and the lack of other clinically applicable means to perform such measurements. While the EPR methods do require the intravascular injection of a water soluble probe or prior local implantation of a particulate probe in the tissue of interest, there is no locally invasive procedure needed at the time of measurement. The particulate probes typically are introduced into tissues using a small gauge needle (21–25 gauge), often several days prior to measurement, in order to minimize trauma and allow for healing and incorporation of the material into the tissue prior to pO_2 measurement. An advantage of the particulate approach is that once the probes have been introduced, they may be used to make repeated measurements at the same location without the need to introduce additional material or wait for introduced material to be cleared. This characteristic is particularly useful for the monitoring of tissue oxygenation during the development of disease or in response to therapy, as needed for the optimal treatment of solid tumor cancers and peripheral vascular disease.

We carry out EPR oximetry in human subjects using India ink as the EPR-sensitive oxygen reporter [4, 5]. India ink has a long history of clinical use as an anatomic marker for surgery and radiotherapy and, fortuitously, the suspended carbon black contains stable radical species at sufficient concentrations with EPR spectra that are highly sensitive to the presence of oxygen. The presence of molecular oxygen leads to a decrease in the EPR relaxation time of these radicals, and an associated oxygen dependent broadening of the observed absorption spectrum. This broadening can be directly and quantitatively related to the tissue pO_2 and described by use of an established monotonic calibration curve. India ink and other EPR oximetry particulate probes are highly sensitive to changes occurring at low levels of oxygen, making them very well suited to the study of ischemic diseases and tissue hypoxia.

2 Materials and Methods

The ongoing clinical EPR oximetry measurements are performed using an India ink formulated as a suspension of Printex-U carbon black (200 mG/mL) in a 0.9% saline solution with carboxy methyl cellulose as a suspending agent [6]. For these studies 20–50 μL of ink was injected directly into the tissue of interest using a 21 gauge needle. Occasionally, this injection was preceded by the application of local lidocaine anesthesia. For measurements in normal subcutaneous tissue, the ink was injected approximately 2 mm below the surface of the skin; for measurements in tumors, the depth varied depending on the specific anatomy with a range of 2–10 mm below the surface of the skin. At least one day was allowed for healing and incorporation of the ink into the tissue prior to oxygen measurement.

All of the measurements were made using a low frequency (1.2 GHz) clinical EPR spectrometer specifically designed for human applications [7, 8]. The spectrometer is equipped with a number of ancillary patient positioning devices, including an insertable bed which facilitates measurements of subjects in a lying position and an adjustable chair which is used for foot measurements. Throughout all measurements, the surface temperature of the subject is monitored using a thermocouple and a warm air supply is adjusted to maintain a temperature of 37°C.

The parameters used for EPR data acquisition varied in accordance with the properties of the observed signal. In general, parameters were chosen to avoid instrumental distortion of the EPR spectral shape. Spectra were individually recorded, averaged, analyzed using non-linear least squares spectral fitting, and the fitted linewidth was converted to pO_2 using the previously determined oxygen calibration.

3 Results

The response of tumors to cytotoxic therapies, especially ionizing radiation, is critically dependent on pO_2 [9]. In vitro studies indicate that cells in hypoxic environments are approximately 3-times less sensitive to radiation than cells that are well oxygenated. Accordingly, tumor hypoxia is a major limiting factor in the application of radiation therapy and its efficacy. It is also important to recognize that tumor pO_2 is not static, especially during the course of treatment when changes in O_2 consumption, interstitial pressure, and perfusion are expected. If available, direct knowledge of tumor pO_2 could be used to optimize treatment on an individual basis through the application of drugs or procedures that increase tumor oxygenation and/or the optimizations of both temporal and spatial patterns of irradiation to maximize the therapeutic ratio.

Building off of instrumental and methodological developments and previous successes in animal model systems, we are now pursuing the development of

EPR oximetry in a clinical setting to meet this need and demonstrate the feasibility of these measurements within the clinical setting. We have performed measurements on tumors in 10 subjects, including 7 with melanoma lesions and metastases, and others with basal cell, soft-tissue sarcoma, and lymphoma tumors. For several subjects, measurements in more than one tumor, or more than one site, have been possible. The distribution of measurement sites is shown in Fig. 1a, which illustrates the capability of making measurements with the current in vivo EPR spectrometer at a wide variety of locations, from head to toe.

We have previously described the ability of the EPR measurements to measure baseline levels of tumor pO_2 and the response to the inspiration of 100% O_2 [10, 11]. In both of these instances, including melanoma and lymphoma tumors, baseline pO_2 values were observed to be quite low (13 and 4 mmHg, respectively) and the application of inhaled oxygen led to dramatic increases in tumor pO_2. Similar measurements have been made with additional subjects, and serial measurements during the course of radiation treatment have been performed. One such set of serial measurement was performed at 2 sites within separate metastatic melanoma tumors during a course of radiation treatment where a total of 36 Gy was applied using 6 Gy $\times$ 6 doses (Fig. 1b). The tumors were located in the upper right scalp and on the right side of the neck just below the ear. Similar pO_2 levels were observed at each site immediately before and after each fraction, but differences were observed between the sites and during the course of treatment. In the tumor located in the neck, consistently low, nearly anoxic, values were recorded with a small upward trend as the therapy progressed. In the scalp, considerably higher values of pO_2 were observed, ranging from approximately 3–10 mmHg.

It is especially important to note that in vivo EPR oximetry has been used successfully in the clinical setting to make repeated non-invasive direct measurements of tumor pO_2. We have observed that the tumor pO_2 values have varied among the patients studied and over the courses of treatment and that different responses of tumor pO_2 to increased fractions of inhaled oxygen are observed. Based on the measurements to date, we believe that it is feasible that in vivo EPR oximetry could be used to monitor tumor pO_2 in the clinical setting and guide the optimal application of strategies to enhance tumor oxygenation at the time of treatment.

Peripheral Vascular Disease (PVD) is a major cause of morbidity and mortality in diabetics, where a local low tissue pO_2 due to poor perfusion can lead to the development of chronic wounds, which often necessitates amputation. The direct measurement of tissue pO_2 would facilitate the rational development of treatments for PVD and could be applied on individual bases to monitor the development of the disease and guide the application of interventions. The development of in vivo EPR oximetry of subcutaneous tissue aimed at assessment of PVD has begun with measurements in healthy subjects to develop the necessary procedures and to observe the short- and long-term oxygen dynamics present in a controlled population. We have made

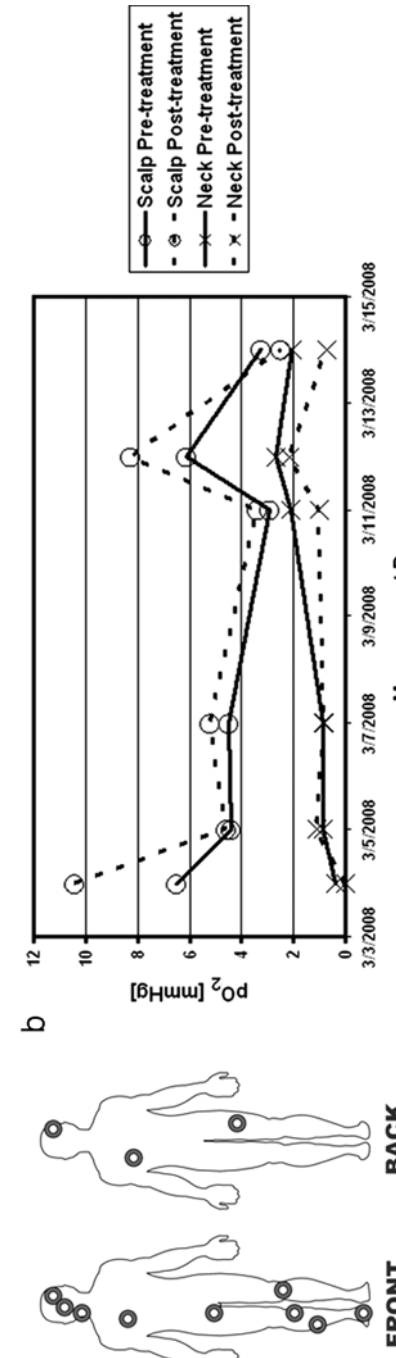

Fig. 1 (**a**) Tumor pO_2 has been measured for 10 volunteers with different tumor locations from head to toe. (**b**) Tumor pO_2 was monitored in melanoma metastases at two sites, in the scalp and neck, during the course of radiation treatment. Spectra were recorded immediately before and after each fraction while the patient inspired room air. These results indicate hypoxic environments that vary on a day-to-day basis, but show little acute response in pO_2 due to radiation

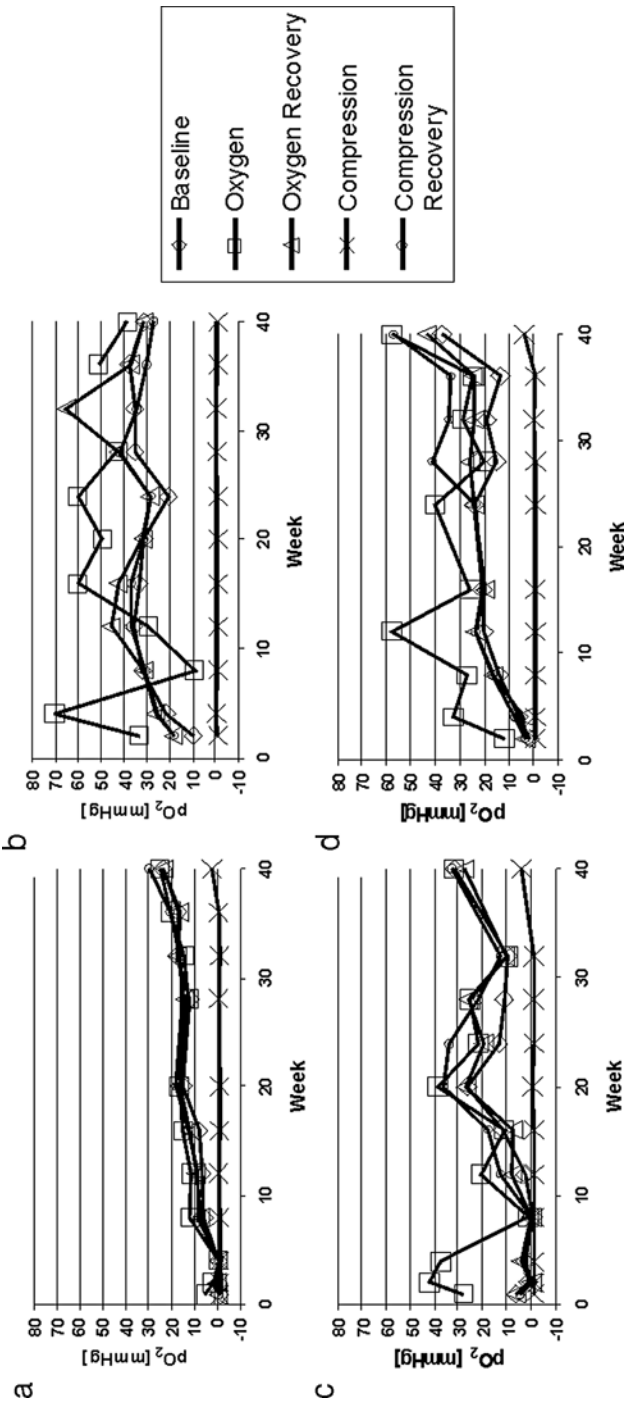

Fig. 2 Tissue pO_2 in the dorsal surface is shown for baseline, inspired O_2, recovery following O_2, compression, and compression recovery periods. For all subjects (**a**–**d**) the baseline O_2 values rise gradually in the weeks after ink injection. Baseline and recovery values are consistent, compression results in anoxia, and O_2 generally leads to increased tissue pO_2

measurements at 14 sites in 9 healthy volunteers, dating back to Oct. 2002. The earliest studies have been described previously [4, 8, 10, 11]. In the current studies, measurements of pO_2 are being performed on a monthly basis at both the dorsal and plantar surfaces of the foot under baseline conditions, as well as with inhalation of increased oxygen and temporary interruption of perfusion of the tissue. Consistent with prior measurements, we have observed that there appears to be a period of decreased pO_2 in the weeks following injection, with a gradual increase back to values near 20–30 mmHg. The nature of this apparent decrease is a matter of ongoing investigation. In all studies, we have consistently observed narrowing of the EPR signal following interruption of perfusion, consistent with consumption driving tissue pO_2 to near anoxic values. Similarly, we see general, but less consistent, increases in tissue pO_2 following the administration of inhaled O_2. Following each of these interventions, we generally observe tissue pO_2 returning to the baseline levels. These patterns of oxygenation are demonstrated in the data included in Fig. 2, which describes measurements in the dorsal surfaces of the feet of 4 of the most recent research subjects.

4 Conclusions

We have demonstrated that in vivo EPR oximetry can provide repeated, direct measurements of absolute pO_2 of tumors and other tissues in human subjects and that the measurement procedure is compatible with clinical practice. In tumors, where pO_2 is a key factor in the efficacy of radiation therapy, preliminary measurements showed that increased pO_2 following inspiration of O_2 varies among patients and tumor pO_2 changes during the course of radiation therapy. Measurements in subcutaneous foot tissue were performed to develop procedures that could be used to assess pO_2 in the treatment of PVD and chronic wound care. These measurements have demonstrated the response of tissue pO_2 to the delivery of O_2 and ability to perform measurements at the same site over long durations for disease monitoring.

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