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Minireview

Bench-To-Bedside Oximetry for Real-Time Monitoring of Tumor Po_2: A Critical Parameter Which Influences Radiotherapeutic Outcome

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Abstract

Tumor hypoxia (pO_2; partial pressure of oxygen < 10 – 15 mmHg) plays a critical role in radio-resistance and promotes the development of aggressive tumor phenotypes. Furthermore, tumor hypoxia is dynamic and varies with tumor type, stage and as a consequence of ionizing radiation and other therapies. In spite of its profound effect on treatment outcome, tumor pO_2 has been sub-optimally exploited in radiation oncology. Current radiotherapy plans do not take into account specific temporal changes in individual tumor pO_2 levels due to lack of appropriate oximetry techniques, and therefore potentially may be suboptimal. In particular, hypofractionated treatments are increasingly used with large doses (4 - 20 Gy) of ionizing radiation that may have different effects on the levels of oxygen in individual tumors during the course of treatment. Real-time monitoring of tumor pO_2 might make it feasible to improve the outcome by scheduling fractions at times of increased tumor pO_2. Such tumor oxygen guided treatment protocols can only be accomplished by oximetry techniques that can provide accurate serial measurements of tumor pO_2 throughout the course of therapy.

We have pioneered *in vivo* EPR oximetry using micro-particulate oxygen-sensing probes for real-time monitoring of tissue pO_2 in superficial (<10 mm) as well as deep-sited tumors, repeatedly and accurately, for clinical applications. Our goal is to improve treatment outcome by providing the information about dynamic tumor oxygen levels so that irradiations can be scheduled when the tumors are better oxygenated either with or without oxygen enhancing interventions e. g. pre-irradiation, carbogen (2 - 5% CO_2 balance O_2) inhalation, hyperthermia, and anti-angiogenic treatment. Temporal changes in tumor pO_2 can also be used as a prognostic marker to predict efficacy, identify responders and non-responders, and individualize therapy. An overview of *in vivo* EPR oximetry, pre-clinical results and the current status of clinical oximetry are briefly described to highlight the potential advantages of EPR oximetry in radiation oncology.

INTRODUCTION

Radiotherapy is an important therapeutic modality for both curative and palliative cancer treatment with nearly 50% of cancer patients undergoing radiotherapy at some point during their illness. The level of oxygen in solid tumors is one of the key pathological parameters that can affect the outcome of treatment. It has been shown that radiosensitivity decreases by a factor of up to three when the pO_2 declines from radiobiologically normoxic levels (>15 – 20 mmHg) to profound hypoxia [1,2]. Several animal and human tumor xenograft studies indicate that acute hypoxia due to transient cessations in microregional blood flow, in addition to chronic hypoxia arising from limitations in oxygen diffusion, may be a significant cause of radiation resistance [3,4]. Tumor hypoxia is also implicated in other aspects of tumor development including genomic instability, aggressive phenotypes, metastases, and loss of apoptosis [3,5-7]. In some cases, one-time measurement of tumor pO_2 prior to treatment can be a good predictor of the outcome. A poor prognosis of patients with pre-treatment tumor pO_2 of less than 10 mmHg has been reported for tumors of various origins, including soft tissue sarcoma, head and neck cancer, cervical cancer and esophageal cancer [8-12]. The single assessment of tumor pO_2 in these studies was carried out using invasive electrode techniques or indirect markers of tissue hypoxia (e. g. pimonidazole staining). However, a single measurement cannot provide the crucial temporal information necessary to guide the treatment as the

tumor pO_2 will vary in a complex time-dependent manner during the course of radiotherapy.

The temporal changes in tumor pO_2 can also be induced by radiation through a combination of several factors such as, cell killing, direct vascular damage, and change in interstitial fluid pressure [13,14]. In experimental tumors, an increase or decrease in tumor pO_2 after single small or large doses of radiation has been observed [15-17]. Consequently, it is very desirable to be able to measure tumor pO_2 accurately and repeatedly over the course of radiotherapy. Such information will be extremely useful to (i) evaluate and optimize the effectiveness of methods being developed to increase tumor pO_2 such as carbogen inhalation and anti-angiogenic therapies, and (ii) improve treatment outcomes by reducing the levels of hypoxia and scheduling irradiations when the tumors are well oxygenated. The measurements can be achieved using *in vivo* EPR oximetry, which provides repeated measurement of absolute tumor pO_2 at one or more sites simultaneously [18-20]. We have successfully implemented *in vivo* EPR oximetry using small (microcrystalline) probes for discrete measurement of tumor pO_2 at one or more sites to investigate the effect of radiotherapy and hyperoxic adjuvant approaches on tumor pO_2 with a goal to optimize treatment outcome [15,21-26].

In vivo EPR oximetry for repeated measurement of tumor pO_2

The basis of EPR oximetry is the paramagnetic nature of molecular oxygen (O_2), which therefore affects the EPR spectra of other paramagnetic materials in its vicinity by altering their relaxation rates [18-20,27]. The magnitude of this relaxation effect is directly related to the amount of oxygen that is present in the environment of the paramagnetic materials (oximetry probes). Accordingly, the line width of the EPR spectrum of an oximetry probe, when injected into a tissue of interest, provides a direct measurement of tissue pO_2. Several oximetry probes have been developed with varying sensitivities to oxygen, including the crystals of lithium phthalocyanine (LiPc) and its analogs [28-30]. These crystals are metabolically inert and very sensitive for measuring low levels of oxygen, which makes them especially suitable for pO_2 measurements in tumors [27,28,31]. For clinical applications, these oximetry probes have been encapsulated in oxygen-permeable biocompatible and inert polydimethylsiloxane (PDMS) polymer or Teflon AF2400 [31-35]. *In vivo* EPR oximetry requires a one-time implantation of the oximetry probe (minimally invasive procedure using 25-23 gauge needles or during surgery for other procedures) but all subsequent procedures for pO_2 measurement are entirely noninvasive and can be repeated as often as desired (from few seconds to several weeks). A surface-loop resonator is positioned over the tissue implanted with the oximetry probe and by the use of an appropriate combination of an exciting frequency (1200 MHz for L-band) and a magnetic field (400 Gauss) [36]. The scanning of the magnetic field produces a characteristic EPR signal. By using an appropriate calibration of the oximetry probe used, the line width of the EPR signal provides a sensitive measurement of tissue pO_2. In order to assess the heterogeneity in pO_2, oximetry probes can be implanted at different sites within a tumor and a simultaneous measurement of pO_2 from each implant can be carried out using magnetic field gradients (multi-site EPR oximetry) [37-39].

In vivo EPR oximetry offers several unique capabilities and advantages compared to other approaches:

(i) A direct measurement of actual oxygen content in a tissue of interest, whereas indirect techniques such as BOLD (blood oxygen-level dependent) MRI and NIR (near-infra-red) derive oxygen information from hemoglobin saturation in the vasculature.

(ii) The measurements provide quantitative pO_2 data (techniques such as misonidazole or EF5 provide information on the occurrence of hypoxia but usually do not provide quantitative pO_2 information).

(iii) The measurements can be made continuously and repeatedly as desired, without a confounding influence of prior measurements or a reduction in sensitivity.

(iv) The oximetry probes are metabolically inert and therefore do not perturb the tissue microenvironment including oxygen content (electrochemical techniques such as Eppendorf are invasive and require the consumption of oxygen for pO_2 assessment).

(v) Tumor pO_2 measurements can be made up to a depth of 10 mm from the surface using direct implantation and detection of the particulate oximetry probes.

(vi) Implantable oxygen sensors (ImOS) can be used for pO_2 measurement at depths ranging from 3 mm to greater than 20 cm with excellent detection sensitivity. The particulate oximetry probes are loaded on the sensory tip of the implantable resonators.

(vii) Particulate oximetry probes are deposited via a 23 - 25 gauge needle. ImOS can be implanted via a similar catheter and incision to position the subcutaneous coupling loop, or carried out in conjunction with a scheduled surgery. All subsequent measurements are entirely noninvasive.

(viii) There is no other technique available at present to make repeated measurement of absolute tumor pO_2 without the need to reintroduce the probe for each measurement.

We have extensively used EPR oximetry to study the temporal variations in tissue pO_2 in a wide range of pathologies including experimental and human xenograft tumors, muscle, heart, brain, kidney, and liver in animal models [15,18,21-26,33,40-50]. Recent preclinical and clinical oximetry results are briefly described below.

Experimental and human xenograft tumor pO_2 during radiotherapy and/or hyperoxic challenge

Using *in vivo* EPR oximetry, we have serially measured ectopic and orthotopic tumor pO_2 and the results indicate that

(i) The pre-treatment (baseline) pO_2 varies with the tumor type and size [15,21,24,26,51].

(ii) The extent and timing of post-irradiation changes in pO_2 depends on the tumor type and radiation dose [15,21,25].

(iii) The outcome of radiotherapy can be enhanced if the tumors are irradiated at the time of an increase in tumor pO_2 compared to when they are hypoxic [23,25].

(iv) A significant increase in tumor pO_2 may be observed when inhaling carbogen (5% CO_2 balance O_2). However, not all the tumors response to carbogen (inter and intra-tumor variability) and also the magnitude of increase in pO_2 usually declines with tumor growth over days [15,21,24,25].

(v) Anti-angiogenic approaches such as metronomic chemotherapy [52-54] can significantly increase tumor pO_2. Therefore, a serial measurement of pO_2 is necessary to identify therapeutic window during which the tumors are oxygenated for effectual combination with radiotherapy [46,51,55]. However, the efficacy of such treatment varies with the tumor type and the chemotherapeutic agent (unpublished results).

We are currently using multiple site ImOS to simultaneously measure orthotopic human xenograft glioma and contralateral brain pO_2 during hyperoxia with carbogen inhalation and metronomic chemotherapy using gemcitabine. The tissue pO_2 of the contralateral brain reflects the global changes in pO_2, allowing us to differentiate between those and glioma specific changes in pO_2. The ImOS are assembled from enameled copper wires (thickness: 0.15 mm) and consists of a coupling loop (8 - 10 mm diameter) and transmission lines (3-mm length, i. e. depth from the surface, but capable of being any length) with sensory loops (or tips, 0.2 - 0.3 mm diameter) loaded with a few LiPc crystals [22,24,35,56], Figure 1. The entire assembly is then coated with oxygen-permeable and biocompatible Teflon AF2400 [35]. For intracranial pO_2 measurements, the transmission lines with sensory loops are gently inserted into the brain tissue while the coupling loop remains on the top of the skull under the skin. The coupling loop is inductively coupled with an external surface loop resonator of L-band EPR spectrometer for pO_2 measurements. ImOS with single or multiple sensory loops and varying length of the transmission lines are shown in Figure 1D. Such ImOS designs are suitable for single site or multiple site measurements in deep sited tumors, e. g. head and neck or prostate cancer.

We are currently pursuing an Investigational Device Exemption (IDE) from the FDA for clinical use of ImOS for pO_2 measurements. The mean area at the surface of each sensory loop is estimated to be 0.03 - 0.07 mm^2. EPR oximetry therefore samples a region that includes several capillaries and a region that spans the local heterogeneous tumor structure [18,24,57]. Tumor pO_2 measurement at more than one site using multi-site EPR oximetry is expected to resolve any gradient in pO_2 that may exist across a tumor. The EPR spectrum acquired by a two-probe (sensory loop) ImOS in a mouse brain using multisite EPR oximetry is shown in Figure 2A. The temporal changes in glioma and contralateral brain pO_2 measured simultaneously before and during carbogen inhalation are shown in Figure 2B. First, a baseline pO_2 was measured in the anesthetized mouse breathing 30% O_2 (30 min) and then the mouse was allowed to inhale carbogen (25 min) and the breathing gas was again switched back to 30% O_2 for 15 min.

The mean (±SD) baseline pO_2 of the contralateral brain and U251 glioma were 66.0±2.9 and 15.0±0.8 mmHg respectively, which significantly increased during carbogen breathing. In these experiments, the baseline glioma pO_2 and response to carbogen varied among the animals. The typical changes in the contralateral brain and U251 glioma pO_2 in two animals measured repeatedly for 5 consecutive days are shown in Figure 3. A significant increase in contralateral brain pO_2 was evident in both animals. However, the extent of the increase in pO_2 was different between the animals and also varied over days. A minimal change in the pO_2 of the glioma was observed in one of the animals with carbogen inhalation (Figure 3A). On the other hand, a significant increase in the glioma pO_2 occurred in another animal, but the response to carbogen quickly declined by day 3 with no change in pO_2 on days 4 and 5. These results indicate a considerable variation in response to carbogen inhalation between the animals as well as over days in the same animal. Consequently, a real-time monitoring of tumor pO_2 is necessary to assess day to day variation in the response to carbogen inhalation. This information will be especially useful to identify the tumors that are oxygenated and therefore will be sensitive to radiotherapy.

Several anti-angiogenic approaches, such as bevacizumab or metronomic chemotherapy, are being investigated to normalize the chaotic and inefficient vasculature to restore blood flow and improve drug delivery in the tumors [52-54,58]. However, in order for these approaches to be successful, it is desirable to directly measure their effect on tumor vasculature or identify surrogate markers that can be repeatedly monitored during the treatment. We anticipate that the restoration of blood flow into the tumors should increase pO_2, and therefore if measured repeatedly, can be used as a marker to assess the effectiveness of anti-angiogenic treatments. To test this hypothesis, we are currently investigating the effect of metronomic chemotherapy

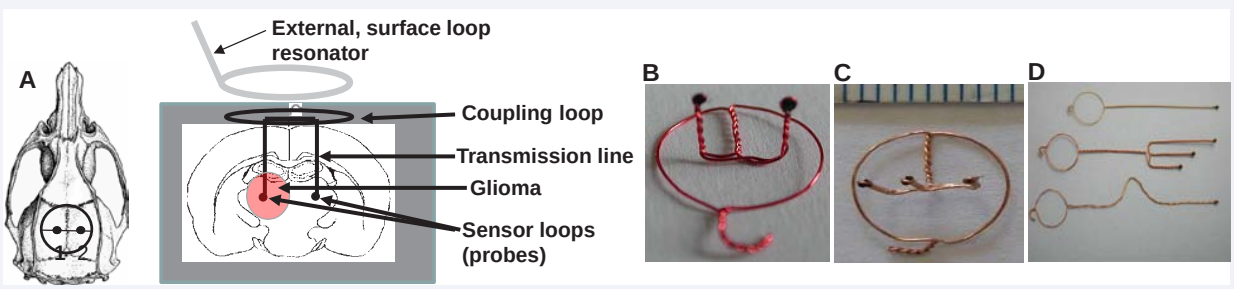

Figure 1 Implantable oxygen sensors (ImOS) with multiple sensory tips. (A) Schematic of a two probe (sensor loops) ImOS coupled to an external surface-loop resonator for pO_2 measurements at two sites in the mouse brain. (B) A two-probe resonator. (C) A three-probe resonator for pO_2 measurements at two sites in the glioma and one site in the contralateral brain. (D) Single and multiple sensory loop ImOS with different lengths of the transmission lines for pO_2 measurement at different depths.

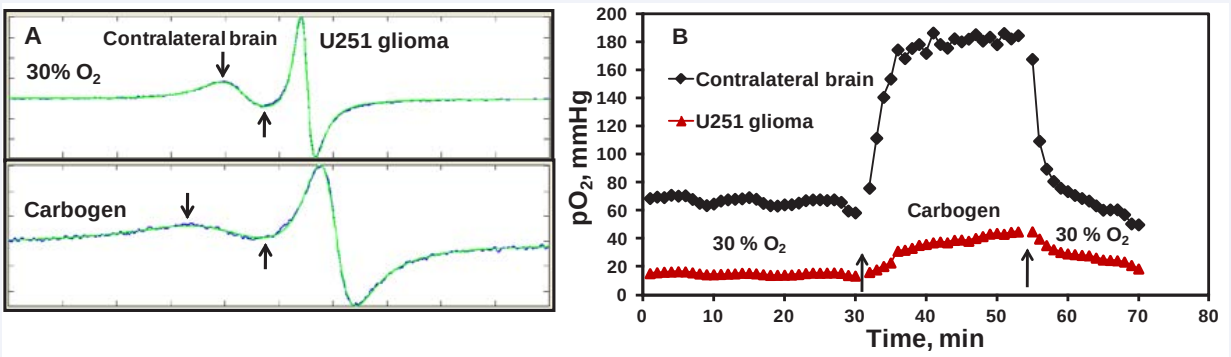

Figure 2 Measurement of pO_2 values in a tumor-bearing mouse brain. (A) EPR spectra acquired using ImOS in the brain of an anesthetized (1.5% isoflurane) mouse breathing 30% O_2 and carbogen. (B) Temporal changes in the pO_2 during 30% O_2 and carbogen breathing and when the gas was switched back to 30% O_2. The EPR spectrum (blue) was fitted (green) by a MATLAB program to determine the line width (indicated by arrows) which was converted to pO_2 using the calibration of the oximetry probe used in this study.

with gemcitabine on the pO_2 of human xenograft U251 glioma, Figure 4. The dose and schedule of gemcitabine in this study were derived from the various metronomic treatment reported in pre-clinical studies [59-61]. In these experiments, the untreated U251 gliomas were hypoxic with a mean pO_2 of 6 - 9 mmHg on day 1. No significant change in the pO_2 of the control group was observed during glioma growth for five consecutive days. A significant increase in the glioma pO_2 only on day 3 was evident in the mice treated with a single dose of 150 mg/kg gemcitabine on day 1. On the other hand, glioma pO_2 increased significantly from day 3 - day 5 when the mice were treated with metronomic gemcitabine (30 mg/kg x 5 and 10 mg/kg x 4). Such increases in glioma pO_2 are expected to significantly improve treatment outcome when combined with radiotherapy. However, the profile of temporal changes in glioma pO_2 varied with different gemcitabine dose/schedule, which highlights the need for repeated measurements of glioma pO_2 for efficacious combination of such approaches with radiotherapy.

Bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor (VEGF), has been approved by FDA as a single agent to treat glioma patients with progressive disease after prior therapy. This antibody has shown anti-angiogenic effect with a transient normalization of vasculature in the gliomas [54,62,63]. However, lack of methods to repeatedly measure and follow vessel normalization has been a rate limiting step in rationally translating these approaches into the clinic and in developing efficacious protocols for combination therapies. A real-time monitoring of tumor pO_2 during hyperoxic or anti-angiogenic approaches will provide critical information on tumor oxygenation and/or vessel normalization that can be used to predict outcome, identify non-responders and efficiently combine these approaches with radiotherapy to improve therapeutic outcome.

Current status of clinical EPR oximetry

We have developed an L-band (1.2 GHz) EPR spectrometer using a permanent magnet with a gap of 50 cm between the poles of the magnet to comfortably position most human subjects for oximetry measurements [36,64]. The suitability of the clinical EPR system for pO_2 measurements in superficial tumors (< 10 mm depth) using India ink as an oximetry probe has been demonstrated for locations ranging from the feet, to the anterior and posterior surfaces of the torso, and scalp [18,64-68]. To date, we have performed repeated pO_2 measurements in superficial tumors at different sites of 14 patients. The tumors varied considerably in their baseline pO_2 (0 to 10 mmHg). Furthermore, the response to breathing enriched oxygen varied considerably between the patients with a significant increase in pO_2 in some tumors while others had minimal or no change in pO_2 from the baseline. These results demonstrate the feasibility of *in vivo* EPR oximetry in the clinical setting to make repeated noninvasive direct measurements of tumor pO_2. Importantly, variability in baseline tumor pO_2 and response to enriched oxygen signify the need for a direct measurements of pO_2 during hyperoxic approaches to confirm, if any, increase in pO_2 for effective combination with radiotherapy.

SUMMARY

The extent of the influence of hypoxia in tumorigenesis makes it a critical factor that must be targeted to achieve therapeutic benefit. The availability of oximetry methods that can provide real-time monitoring of tumor pO_2 are key for improving the outcome of radiotherapy by refining the standard protocols (optimize the timing of combined therapy involving radiation and hyperoxic/anti-angiogenic approaches) and by providing patient-specific pO_2 information to individualize therapy (by altering the timing of fractions to maximize therapeutic effect for a specific patient). Extensive preclinical and initial clinical studies have demonstrated the potential of EPR oximetry to fulfill this need. An expected application of clinical EPR oximetry will be in the ARCON trials [69,70]. In these trials, carbogen and nicotinamide is being used to reduce hypoxia, however, no direct measurement of tumor pO_2 was made to confirm whether the tumors indeed have or have not been oxygenated. Direct pO_2 measurements with *in vivo* EPR oximetry will allow the identification of patients who have responded to carbogen breathing and therefore will benefit from radiotherapy. The results can also be used to evaluate the outcomes between the cohorts who did or did not have an increase in tumor pO_2 to correctly validate the trials. In concluding, we anticipate that in vivo EPR oximetry is likely to play a crucial role in radiation oncology for prognosis and identify responders to individualize therapy.

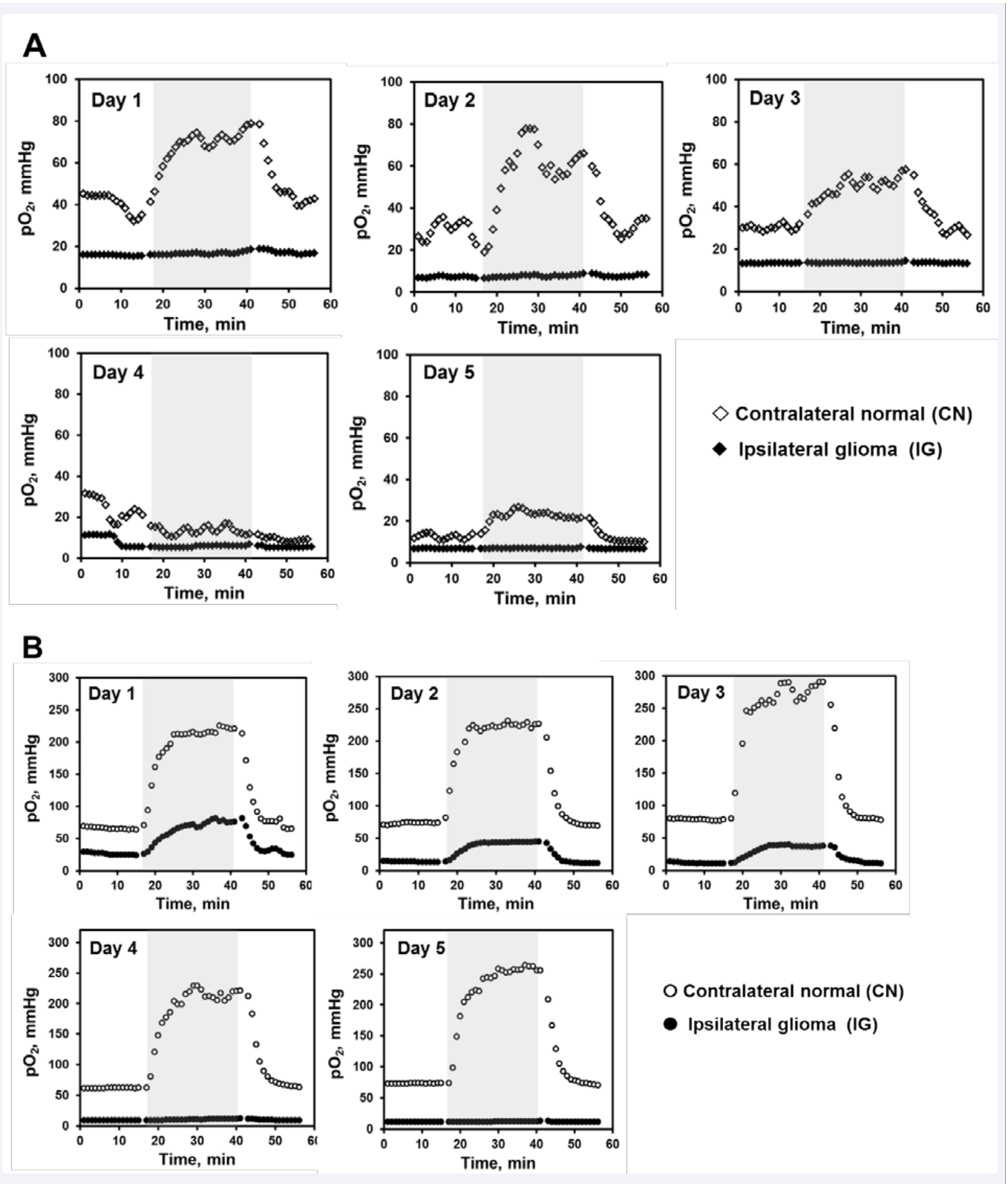

Figure 3 Dynamic changes in the contralateral normal brain (CN) and ipsilateral U251 glioma (IG) pO_2 measured by *in vivo* EPR oximetry before, during and after carbogen inhalation in two mice (A) mouse #1, (B) mouse #2 for 5 consecutive days. The animals were anesthetized by using 1.5% isoflurane with 30% O_2 and a baseline pO_2 was measured for 15 min and then the inhaled gas was switched to carbogen for 25 min (shaded area) to investigate the efficacy of hypercapnic hyperoxia. The gas was again switched back to 30% O_2 and the pO_2 measurements were continued for another 10 min on each day.

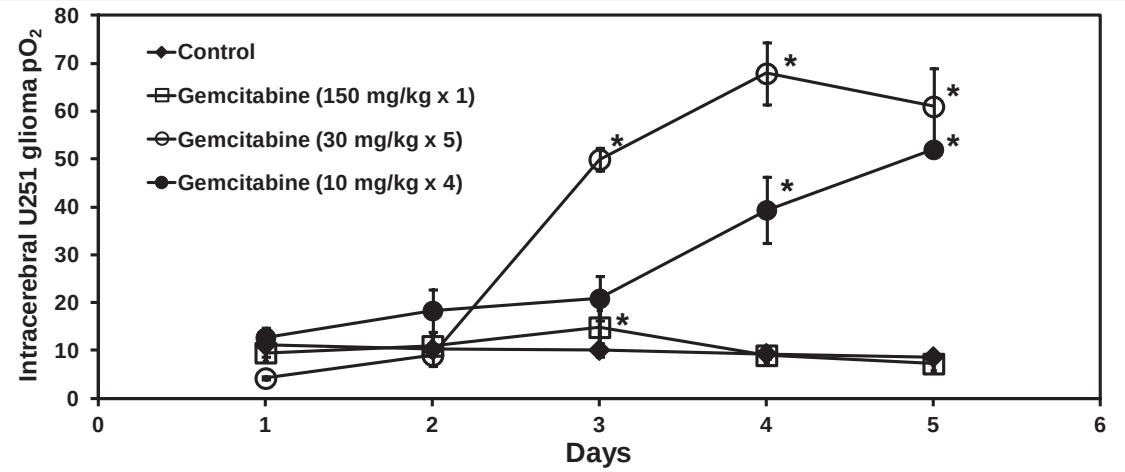

Figure 4 Intracerebral U251 glioma pO2 of the control mice (vehicle alone), and the mice treated with single dose of 150 mg/kg (day 1), 30 mg/kg x 5 (day 1 - 5), and 10 mg/kg x 4 (day 1 - 4) gemcitabine. The gemcitabine was administered intravenously (i.p.) to the athymic nude mice bearing intracerebral U251 tumors. *, $p < 0.05$ vs. day 1; Mean±SEM, n = 2 - 3.

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