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Synergistic combination of hyperoxygenation and radiotherapy by repeated assessments of tumor pO₂ with EPR oximetry

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Abstract

The effect of hyperoxygenation with carbogen (95% O₂ + 5% CO₂) inhalation on RIF-1 tumor pO₂ and its consequence on growth inhibition with fractionated radiotherapy is reported. The temporal changes in the tumor pO₂ were assessed by *in vivo* Electron Paramagnetic Resonance (EPR) oximetry in mice breathing 30% O₂ or carbogen and the tumors were irradiated with 4 Gy/day for 5 consecutive days; a protocol that emulates the clinical application of carbogen. The RIF-1 tumors were hypoxic with a tissue pO₂ of 5 – 9 mm Hg. Carbogen (CB) breathing significantly increased tumor pO₂, with a maximum increase at 22.9 – 31.2 min on days 1 – 5, however, the magnitude of increase in pO₂ declined on day 5. Radiotherapy during carbogen inhalation (CB/RT) resulted in a significant tumor growth inhibition from day 3 to day 6 as compared to 30% O₂/RT and carbogen (CB/Sham RT) groups. The results provide unambiguous quantitative information on the effect of carbogen inhalation on tumor pO₂ over the course of 5 days. Tumor growth inhibition in the CB/RT group confirms that the tumor oxygenation with carbogen was radiobiologically significant. Repeated tumor pO₂ measurements by EPR oximetry can provide temporal information that could be used to improve therapeutic outcomes by scheduling doses at times of improved tumor oxygenation.

Keywords

Carbogen; EPR oximetry; irradiation; tumor oxygenation

INTRODUCTION

Tumor hypoxia leads to therapeutic resistance ^(1–3) and also promotes aggressive tumor behavior and metastases ^(4, 5). Therefore, a significant therapeutic benefit can be achieved by improving tumor oxygenation. Several methods have been developed to increase tumor oxygenation such as by using gases with high oxygen content at normobaric or hyperbaric pressures ^(6, 7), carbogen (95% O₂ + 5% CO₂) breathing, carbogen combined with nicotinamide (8–10), administration of hypoxic-cell sensitizers ^(11, 12), and erythropoietin to improve hemoglobin level ^(13, 14). Although these methods have led to mixed results, it is generally agreed that radiotherapeutic outcome should improve, if an effective strategy is established to reduce tumor hypoxia.

Carbogen (CB) inhalation seems to be the most effective method to enhance the radiation response of experimental tumors ^(12, 15, 16). The clinical ARCON therapy (accelerated radiotherapy with carbogen and nicotinamide) utilizing carbogen with nicotinamide has also

led to promising results⁽⁹⁾, however, the mechanism of hyperoxic hypercapnic gas such as carbogen remains unclear⁽¹⁷⁾. Appropriate oximetry techniques that can provide repeated assessments of tumor pO₂ are necessary to optimize hypoxia modifying methods and efficaciously combine with radiotherapy.

In vivo Electron Paramagnetic Resonance (EPR) oximetry has the ability to provide repeated measurements of tumor pO₂ to test and optimize methods designed to increase tumor oxygenation and demonstrate therapeutic optimization^(18–22). We report the effect of carbogen inhalation on the tissue pO₂ of subcutaneous RIF-1 tumors. The maximum increase in the tumor pO₂ and the time to reach the maximum pO₂ during carbogen inhalation were determined by EPR oximetry. This crucial information was used to investigate therapeutic outcome by irradiating the tumors with 4 Gy/day for 5 days at the time of a maximum increase in the tumor pO₂.

MATERIALS AND METHODS

1. Study design

The effect of carbogen (CB) inhalation on RIF-1 tumor pO₂ was measured by EPR oximetry for 5 consecutive days and the changes in the tumor pO₂, maximum pO₂ (pO₂ max), and the time to reach to maximum pO₂ (T max) were assessed (CB alone group, N = 14). The tumor pO₂ was assessed prior to and during carbogen inhalation and the tumors were irradiated with 4 Gy at T max and these experiments were repeated for 5 consecutive days (CB/RT treatment group, N = 16). In group 30% O₂/RT, the tumors were irradiated with 4 Gy in mice breathing 30% oxygen (30% O₂/RT treatment group, N = 9). In group CB/sham RT, the tumors were treated with sham irradiation (0 Gy) in mice breathing carbogen (CB/sham irradiation group, N = 6). The inspired gas of 30% O₂ (balanced N₂) provides blood pO₂ values in the normal range and therefore this gas mixture is routinely used in our laboratory (18, 19, 23–25).

General methods

Tumor models—The radiation-induced fibrosarcoma (RIF-1) cells were a gift from Dr. J. B. Mitchell's laboratory at the National Cancer Institute. This is a well established subcutaneous tumor model used extensively in our lab^(18, 24). The cells were cultured in RPMI 1640 medium supplemented with 10% FBS, glutamine, and antibiotics. The procedure for tumor inoculation has been reported previously⁽²⁴⁾. Briefly, 18 – 20g female C3H/HEJ mice (4 – 5 weeks age, Charles River Lab, MA) were anesthetized by 1.5% isoflurane with 30% O₂, and a suspension of 2×10^5 cells in 60 – 80 μ l was injected subcutaneously into the left posterior flank. The tumors were allowed to grow for 12 – 14 days and oxygen sensitive lithium phthalocyanine (LiPc, oximetry probe) crystals were implanted into the tumors as described below. The tumor volumes were estimated using the formula: $\pi/6 \times \text{length} \times \text{width}^2$ for six days.

Oximetry probe and EPR (Electron Paramagnetic Resonance) oximetry—LiPc crystals were synthesized in our laboratory; its physicochemical properties and use for tissue pO₂ measurement have been described previously^(26, 27). LiPc has a single sharp EPR line whose width is highly sensitive to pO₂. The EPR spectra reflect the average partial pressure of oxygen on the surface of the crystals. The high density of unpaired spins combined with a narrow intrinsic line width of LiPc allows the measurements of tissue pO₂ using a few crystals (30–50 μ g) with a total diameter of ~ 200 μ m. For LiPc implantations, the mice were anesthetized (1.5% isoflurane, 30% O₂), and two aggregates of LiPc crystals (30–50 μ g each) were implanted into each tumor using 25 gauge needles and wire styluses. In order to simultaneously assess pO₂ at two locations in the tumor, we have used two LiPc

aggregates with appropriate magnetic field gradients to resolve the EPR signals. The magnitude of the gradient required to separate the spectra, while minimizing additional broadening of the EPR lines, depends on the actual line width, size of the implants and the distance between them. In these experiments, the distance between LiPc aggregates was 4 mm to minimize magnetic field gradient and its effect on the EPR line widths. The procedure for multi-site EPR oximetry is described by Smirnov *et al.* ⁽²⁸⁾ and this approach has been used to study pO₂ at multiple locations in the tumors ^(18, 19, 23–25). The tissue pO₂ measured from the two LiPc implants in each tumor were pooled to determine average tumor pO₂ on each day ^(18, 19, 24). The EPR line widths were converted to pO₂ using a calibration determined for the LiPc crystals used in this study. The pO₂ max and T max from each implant were determined, and then averaged to obtain individual mean for each group.

Procedure for carbogen inhalation and irradiation—The mice were anesthetized (1.5 % isoflurane, 30% O₂) 24 hr after LiPc implantation and the baseline pO₂ (day 1) was measured for 20 min in CB group. The breathing gas was then switched to carbogen and oximetry measurements were continued for 60 min to determine the effect of carbogen inhalation on the tumor pO₂. The pO₂ max and T max were determined and used to design radiotherapy (RT) groups. In the CB/RT group, first a baseline pO₂ was measured in mice breathing 30% O₂ and then the mice were allowed to breathe carbogen. The tumor pO₂ was measured for 15 min and then the mice were moved to the irradiation bed of a Varian Linear Accelerator (Clinac 2100C, 6 MeV, 6 cm × 6 cm applicator). In this group, the mice continued to breathe carbogen during irradiation with 4Gy. The beam was focused on the tumor and appropriate lead shields were used to limit irradiation of the normal tissue. The approximate time between the end of the EPR measurements and beginning of the irradiation was 6 – 8 min. The total time to irradiate each tumor with 4Gy took approximately 1.8 min on each day. In 30% O₂/RT group, the tumor pO₂ measurements were continued for 15 min in mice breathing 30% O₂ after baseline measurements and the tumors were irradiated using the procedure described for the CB/RT group. In CB/sham RT group, after baseline and 15 min of carbogen breathing, the tumors received sham irradiation (0 Gy), and then the mice were moved back to the EPR spectrometer for pO₂ measurements for at least 10 min. These experiments were repeated for 5 consecutive days.

The tumor pO₂ measured on day 1 prior to any treatment is termed as baseline pO₂, while the initial pO₂ measured on days 2 – 5 in the first 20 min is termed as pre-treatment pO₂. Similar terms are used for tumor volume measurement in these experiments.

Physiological control and histological analysis—During tumor pO₂ measurements, the body temperature of the animals was monitored using a rectal probe and maintained at 37 ± 0.5°C by keeping the body warm with a thermostatically controlled heated pad and a flow of warm air. The animals were kept warm by an electric heating pad during the transportation to and from the irradiator.

After the last tumor volume measurements on day 6, the animals were euthanized, tumors removed, fixed, and sectioned. Microscopic examination (H & E staining) of the tissue around the implanted LiPc deposits was performed to confirm its location in the tumor.

Statistical Analysis

A paired t-test was used to determine the statistical significance of the changes in pO₂ and tumor volume within the group and an unpaired t-test was used to determine the significance between groups. The paired comparison reduces the effects of animal to animal heterogeneity and eliminates differences of the baseline pO₂. The Chi-Square test was used

to compare the distribution of percentage (%) of tumors with an increase in pO_2 of more than 50% from the baseline during carbogen inhalation when compared with day 1. The tests were two-sided, and a change with $p < 0.05$ was considered significant. All data are expressed as mean \pm SEM; N is the number of animals in each group.

RESULTS

Effect of carbogen inhalation on tumor pO_2

No significant difference in the baseline and pre-treatment tumor pO_2 was observed, in the CB group, Figure 1 and Table 1. There were also no apparent effects of the tumor growth on tumor pO_2 over days. The increase in the mean tumor pO_2 during carbogen inhalation at 1 – 15, 16 – 30, 31 – 45, 46 – 60 min on day 1 – day 5 were significantly higher than the baseline and pre-treatment pO_2 ($p < 0.05$ or $p < 0.01$), Figure 1. These results indicate that the carbogen inhalation provided a significant and consistent increase in tumor pO_2 , with T max at 22.9 ± 3.6 min to 31.2 ± 4.1 min from day 1 to day 5, Table 1. The tumors were irradiated during T max to determine the effect of carbogen or 30% O_2 inhalation on the therapeutic efficacy in the CB/RT, 30% O_2 /RT and CB/Sham RT groups.

The percentage of tumors with an increase in pO_2 of more than 50% from the baseline within 15 min and 30 min of carbogen inhalation on each day are shown in Figure 2. The results indicate a significant decrease in the response of the tumors to carbogen on day 5 as compared to day 1.

Tissue pO_2 and growth of tumors in mice breathing 30% O_2 or carbogen with/without RT

The temporal changes in the tumor pO_2 observed in the CB/RT, 30% O_2 /RT and CB/sham RT groups are summarized in Figure 3 (a – c). No significant change in the baseline and pretreatment tumor pO_2 was observed in the CB/RT and CB/sham RT groups during 5 days of experiments (Figure 3 d & f). However, the pre-treatment tumor pO_2 of 30% O_2 /RT group increased significantly on day 5 as compared to day 1 (Figure 3 e). A significant increase in the tumor pO_2 in CB/RT and CB/sham RT groups was observed at 15 min post carbogen inhalation on days 1 – 5 and days 1 – 4, respectively, as compared to baseline pO_2 (Figure 3 d & f). No such changes in the tumor pO_2 were observed in 30% O_2 /RT group over days (compared to baseline and pretreatment pO_2 , Figure 3e).

In the CB/RT and CB/sham RT groups, the percentage (%) of tumors with an increase in pO_2 of more than 50% from the baseline within 15 min of carbogen inhalation significantly decreased on day 5 as compared with day 1 (Figure 4 a & c), while no such changes were observed in 30% O_2 /RT group, (Figure 4b).

No significant difference in the baseline tumor volume was observed between groups, Table 2. However, irradiation resulted in a significant decrease in the tumor growth in CB/RT and 30% O_2 /RT groups as compared to CB and CB/sham RT groups. Furthermore, a significant decrease in the tumor growth was observed in the CB/RT as compared to 30% O_2 /RT group on days 5 and 6 (Figure 5).

Morphologic examination of the location of LiPc in the tumor

Gross and microscopic examination (H & E staining) of the tissue around the LiPc deposits confirmed that the deposits were in the interstitial compartment of the tumor tissue with no evidence of edema or infiltration of inflammatory cells. Although, some accumulation of red blood cells and necrotic cells around the LiPc deposits was observed in some samples, but these were not caused by the LiPc implants, rather it reflects the normal histological pattern

of the tumor. This is consistent with the previous report on the histological appearance of RIF-1 tumors ⁽²⁹⁾.

DISCUSSION

It is well known that hypoxic cells, as often occur in tumors, are radioresistant and require nearly three times as much radiation to achieve an equivalent cell kill than oxygenated cells ⁽³⁰⁾. Mean tumor pO₂ of less than 10 mmHg have been shown to correlate with treatment failure ^(4, 31–33). Consequently, several approaches to reduce tumor hypoxia have been developed but only a modest success could be achieved, perhaps due to lack of appropriate techniques that can be used to follow the changes in tumor pO₂ during such treatments. With the development of EPR oximetry, it is now possible to repeatedly assess tumor pO₂ to optimize the timing of radiotherapy and efficaciously combine it with other therapeutic modalities. EPR oximetry requires a onetime implantation of the oximetry probe using 23 – 25 gauge needles in a tissue of interest but rest of the measurement procedure is entirely non-invasive and provides repeated assessments of localized changes in the tissue pO₂ during treatments ^(34, 35). Such measurements are not feasible with techniques such as polarographic electrodes or Oxylite as the measurement procedure is invasive and cannot be repeated in the same tumors at different time points (or days).

These results are from our ongoing studies to characterize the changes in tumor pO₂ induced by carbogen, with the aim to enhance radiotherapeutic outcomes by scheduling radiation at times of increases in tumor pO₂. The RIF-1 tumors were hypoxic and the baseline/pretreatment pO₂ were stable during 5 days of repeated measurements in CB, CB/RT and CB/sham groups. A significant increase in the tumor pO₂ was observed within 15 min of carbogen inhalation, with the time to reach the maximum tumor pO₂ at around 22 – 32 min. A similar increase in the tumor pO₂ was observed in the CB/sham group, which remained at an oxygenated level after sham irradiation, Figure 4. These results confirm that in the CB/RT group, the tumors were irradiated when a significant increase in tumor pO₂ occurred during carbogen breathing.

The dynamic response of tumor pO₂ during carbogen inhalation has varied among studies. Bussink ⁽⁸⁾ reported a rapid increase in the tumor pO₂ using Oxylite, with the mean time to reach maximum pO₂ at approximately 3.9 (0.8–14) min and 3.6 (0.7–16) min in E102 and E106 glioblastoma xenografts, respectively. Gu et al. reported a significant increase in subcutaneous mammary adenocarcinoma tumors pO₂ within 8 min of carbogen breathing which gradually increased over the next 12 min ⁽³⁶⁾. Using polarographic electrodes, Thews et al. observed a slow increase in the pO₂ of subcutaneous rat DS-sarcomas over 15 min during carbogen challenge ⁽³⁷⁾. Using EPR oximetry, we have reported a significant increase in the intracerebral F98 tumor and contralateral brain pO₂ within 15 min of carbogen inhalation ⁽³⁸⁾. These results indicate that the time to achieve a significant increase in tumor pO₂ with carbogen seems to depend on the tumor type, tumor location, and possibly tumor size.

Approximately half of the tumors had an increase in pO₂ of greater than 50% from the baseline within 15 min of carbogen inhalation on day 1 to day 4 (Figure 2 and 4) but pO₂ significantly declined on day 5 as compared to day 1. This is likely due to a combination of several factors, such as an increase in interstitial pressure, compromised tumor vasculature with increase in tumor size and increase in necrotic areas in tumor over days ⁽¹⁹⁾.

Carbogen breathing (CB) during irradiation resulted in a significant increase in tumor pO₂ and significant suppression of tumor growth compared to the 30%O₂/RT, CB and CB/sham groups on day 3 to day 6. These results are consistent with the previous study employing

carbogen inhalation with radiotherapy of intracerebral tumors ⁽³⁸⁾. Our results suggest that the observed changes in the tumor pO₂ could be used as a marker to optimize radiotherapy. The pretreatment tumor pO₂ increased significantly on day 5 as compared to day 1 (Figure 3 e) in the 30% O₂/RT group. These results are in agreement with Znati et al. who reported an increase in tumor oxygenation when the total dose of more than 10 Gy were used ⁽³⁹⁾ and our previous reports in which a significant increase in pre-treatment tumor pO₂ was observed on day 4 and day 5 after fractionated radiation (18, 24).

In conclusion, these results provide unambiguous quantitative information on the effectiveness of carbogen in enhancing tumor oxygenation over the course of 5 days to a level that significantly increased tumor response to radiotherapy. Since the time to achieve a significant increase in tumor pO₂ is likely to vary with the tumor type, size, and location, EPR oximetry could be potentially used to repeatedly monitor tumor pO₂ during hypoxia modifying interventions and enhance efficacy by scheduling radiations at times of increases in tumor pO₂. *In vivo* EPR oximetry is currently being tested to measure tissue pO₂ in the foot of healthy volunteers with the goal to diagnose and optimize the treatment of peripheral vascular disease in diabetic patients ⁽²⁰⁾. EPR oximetry is also being tested in patients with superficial tumors undergoing chemoradiation with the aim to optimize outcome by scheduling treatments at times of optimal tumor oxygenation ^(20, 21).

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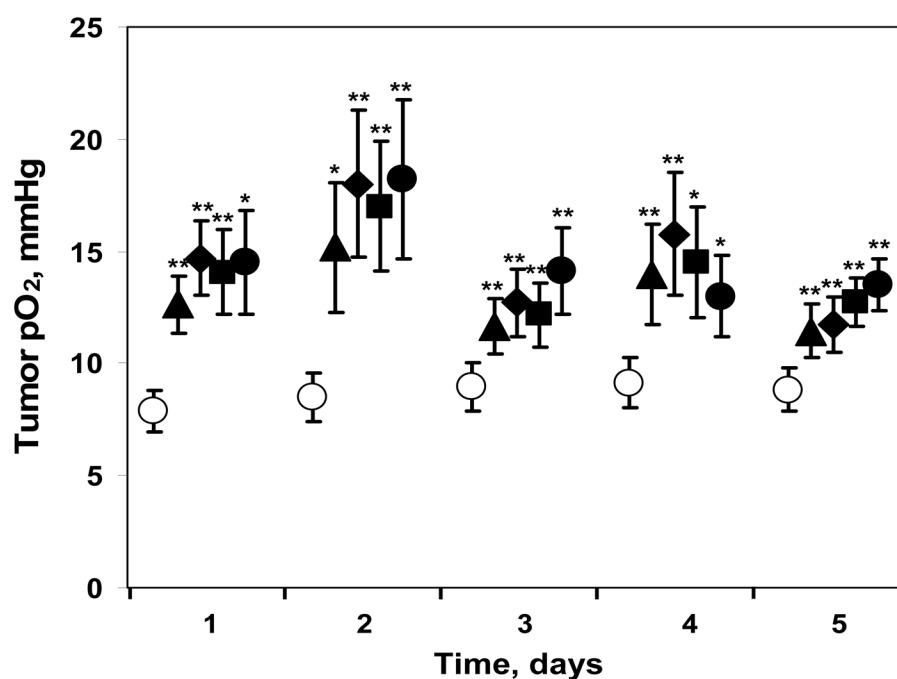


Figure 1.

Average change in the tumor pO_2 during baseline (\circ : baseline pO_2 averaged from 20 min measurements) and response to 60 min of carbogen inhalation (\blacktriangle : 1–15 min; \blacklozenge : 16–30 min; \blacksquare : 31–45 min; \bullet : 46–60 min). * $p < 0.05$, ** $p < 0.01$ compared with baseline and pretreatment pO_2 (paired t-test).

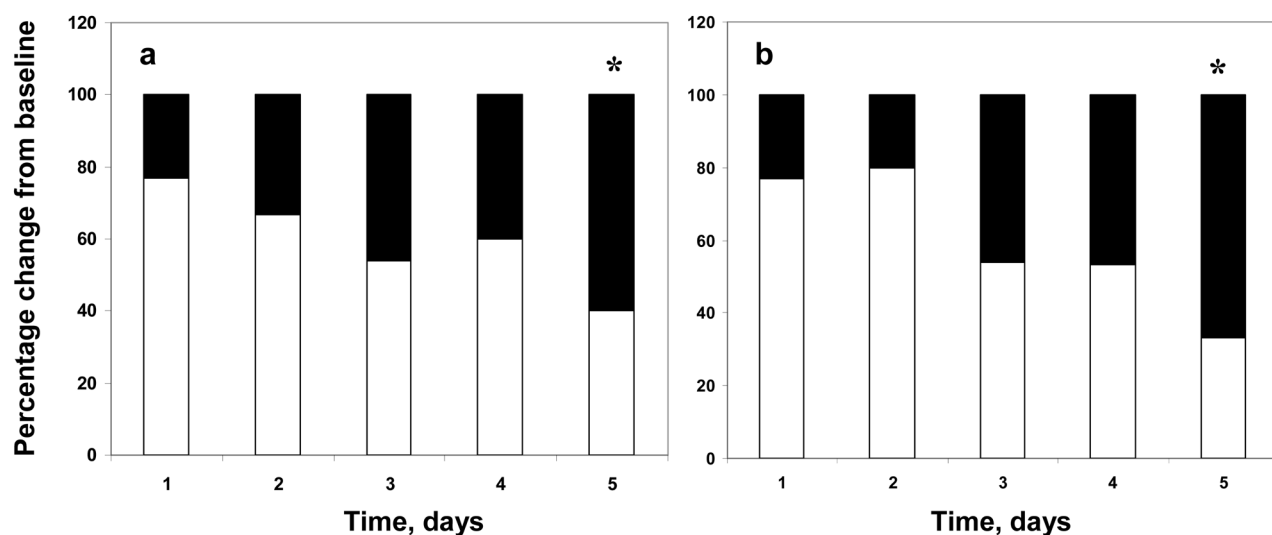


Figure 2.

The percentage change in tumors with pO₂ higher than 50% of the baseline (white column) and less than 50% of the baseline (black column) within 15 min (a) and 30 min (b) of carbogen inhalation in CB group on each day. *p<0.05, compared with day 1 (Chi-Square test).

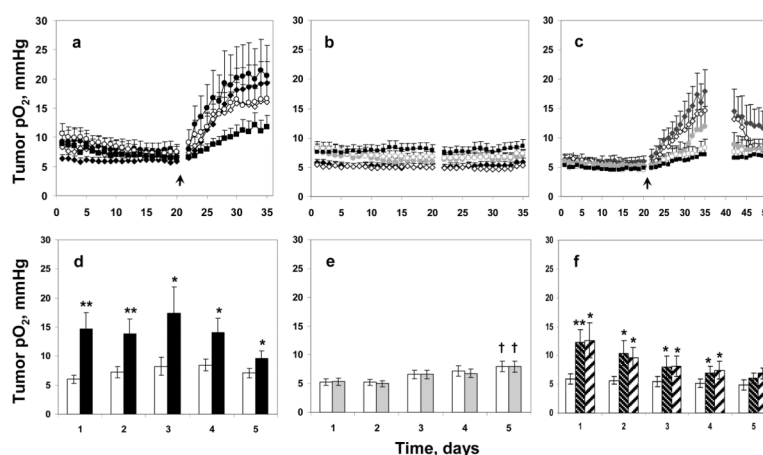


Figure 3.

The time course of mean RIF-1 tumor pO₂ during baseline and pretreatment pO₂ (first 20 min) and during (a) CB/RT, (b) 30%O₂/RT and (c) CB/sham RT on day 1 to day 5 (◆: Day 1; ◇: Day 2; ●: day 3; ○: day 4; ■: day 5). The arrows indicate the time when the inhaled gas was switched from 30% O₂ to carbogen. Averaged baseline and pretreatment (d–f, white column) and the changes in pO₂ in CB/RT (d, black column: averaged for 15 min CB), 30%O₂/RT (e, grey column: averaged for 15 min 30%O₂) and CB/sham RT (f, dense downward diagonal: averaged for 15 min CB and wide upward diagonal: averaged for 10 min CB after irradiation). *p<0.05; **p<0.01, compared with baseline and pretreatment pO₂ on each day (paired t-test), †p<0.05, compared with baseline pO₂ on day 1 (unpaired t-test).

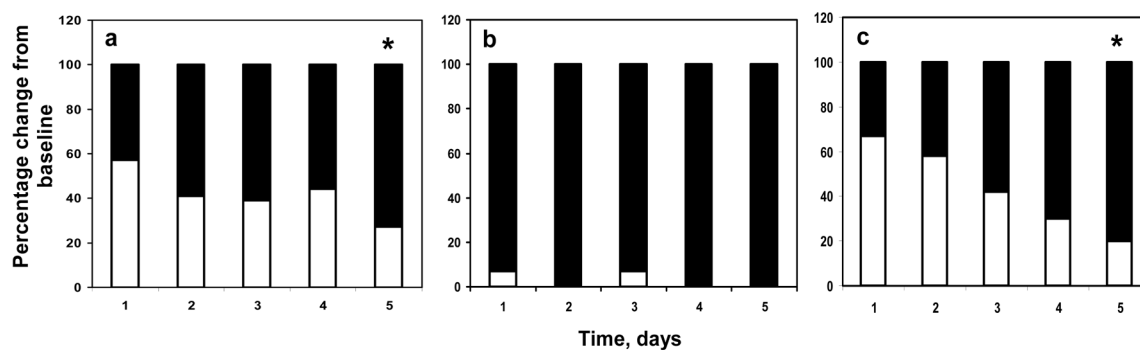


Figure 4.

The percentage change in tumors with pO_2 higher than 50% of the baseline (white column) within 15 min of carbogen inhalation in CB/RT group (a); 30% O_2 in 30% O_2 /RT group (b); and carbogen inhalation in CB/sham RT group (c), on each day. * $p < 0.05$, compared with day 1 (Chi-Square test).

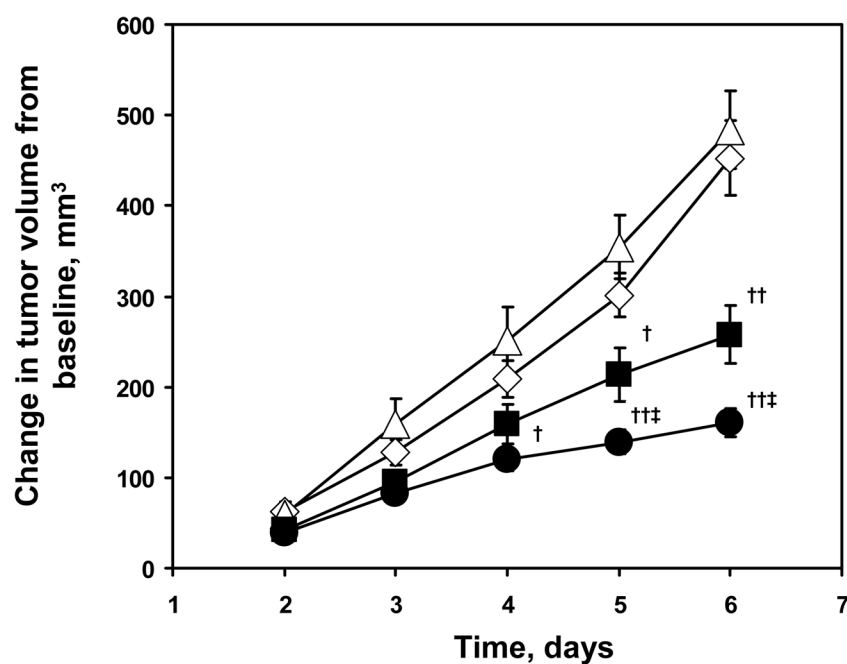


Figure 5.

The change in tumor volume from baseline (day 1) in mice treated with or without RT. ◇: CB; ●: CB/RT; ■: 30%O₂/RT; △: CB/sham RT groups. Data are the mean \pm SEM. † p <0.05, †† p <0.01, compared with the CB group, † p <0.05, compared with 30%O₂/RT group (two-tailed unpaired t-test).

Table 1

Baseline/pretreatment pO₂, maximum pO₂ and time to reach to maximum pO₂ in CB group on 5 consecutive days

Time (days)	pO ₂ base/pre-treatment (mmHg)	pO ₂ max (mmHg)	T max (min)
1	7.9±0.9	22.1±3.6	22.9±3.6
2	8.5±1.1	25.7±5.5	27.1±2.4
3	8.9±1.1	17.2±2.2	28.9±4.4
4	9.1±1.1	20.1±3.4	29.5±3.7
5	8.8±1.0	16.6±1.4	31.2±4.1

Abbreviations: pO₂ base/pretreatment: baseline pO₂/pretreatment pO₂; pO₂ max: maximum pO₂; T max: time to reach maximum pO₂.

Table 2

Absolute baseline/pretreatment tumor volume (mm³, mean \pm SEM) in CB/RT, 30% O₂/RT and CB/sham RT groups on 6 consecutive days

Time (days)	Groups		
	CB/RT	30%O ₂ /RT	CB/sham RT
1	163.2 \pm 16.4	159.6 \pm 22.9	163.5 \pm 8.6
2	204.2 \pm 19.3	201.7 \pm 27.2	222.8 \pm 19.0
3	247.2 \pm 21.2	254.5 \pm 30.2	322.4 \pm 34.5
4	281.9 \pm 23.8	319.2 \pm 38.3	414.0 \pm 44.5
5	303.3 \pm 24.4	372.7 \pm 47.2	517.7 \pm 41.4
6	323.8 \pm 25.9	428.2 \pm 49.9	647.2 \pm 46.7