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MODULATION OF TUMOR HYPOXIA BY TOPICAL FORMULATIONS WITH VASODILATORS FOR ENHANCING THERAPY

Zrinka Abramovic¹, Huagang Hou², Kristl Julijana¹, Marjeta Sentjurc¹, Jean P Lariviere², Harold M Swartz², and Nadeem Khan²

¹Jozef Stefan Institute, Laboratory of Biophysics, Jamova 39, 1000 Ljubljana, Slovenia

²EPR Center for Viable Systems, Department of Diagnostic Radiology, Dartmouth Medical School, Hanover, NH 03755 and Norris Cotton Cancer Center, Dartmouth Hitchcock Medical Center, Lebanon, NH 03756

Abstract

Tumor hypoxia is a well known therapeutic problem which contributes to radioresistance and aggressive tumor characteristics. Lack of techniques for repeated measurements of tumor oxygen (pO₂, partial pressure of oxygen) has restricted the optimization of hypoxia modifying methods and their efficacious application with radiotherapy. We have investigated a non-invasive method to enhance tissue pO₂ of peripheral tumors using topical application of formulations with BN (Benzyl Nicotinate), a vasodilator, and have used EPR (Electron Paramagnetic Resonance) oximetry to follow its effect on tumor oxygenation.

We incorporated 2.5% BN in both hydrogel and microemulsions and investigated the effects on pO₂ of subcutaneous RIF-1 (Radiation Induced Fibrosarcoma) tumors in C3H mice. The experiments were repeated for five consecutive days. The topical application of BN in hydrogel led to a significant increase from a pre-treatment pO₂ of 9.3 mmHg to 11 - 16 mmHg at 30 - 50 min on day 1. However, the magnitude and the time of significant increase in pO₂ decreased with repeated topical applications. The BN in a microemulsion resulted in a significant increase from a baseline pO₂ of 8.8 mmHg to 13 - 18 mmHg at 10 - 50 min on day 1. Experiments repeated on subsequent days showed a decline in the magnitude of pO₂ increase on repeated applications. No significant change in tumor pO₂ was observed in experiments with formulations without BN (vehicle only).

EPR oximetry was successfully used to follow the temporal changes in tumor pO₂ during repeated applications for five consecutive days. This approach could be potentially used to enhance radiotherapeutic outcome by scheduling radiation doses when an increase in tumor pO₂ is observed after topical applications of BN formulations.

1. INTRODUCTION

Hypoxia influences treatment outcome not only through treatment resistance but also by promoting more aggressive tumor behaviors [1, 2]. As a result, several new treatment strategies to improve tumor oxygenation are being investigated to improve the efficacy of therapeutic modalities. These include systemic application of blood flow modifying agents, drugs that can change oxygen consumption, and by increasing the oxygen content in the breathing gas such as by using carbogen (95% O₂ + 5% CO₂) [3-5].

We believe that the measurement of tumor pO₂ during such treatments could be used as a guide to optimize these methods and this information could be used to schedule therapies to

enhance therapeutic outcome. Some very useful information has been obtained by invasive methods (e.g. the polarographic oxygen electrode, or a luminescence optical sensor based on oxygen-quenching) even though they can not be repeated frequently or indirectly by monitoring oxygen availability in the circulatory system (NIR spectroscopy and NMR methods) [6, 7]. With the recent development of *in vivo* EPR oximetry, it is now possible to obtain accurate and repetitive measures of tissue oxygen from the same locations in the tissue [8, 9]. After initial insertion of the paramagnetic oxygen-sensitive probe into the tissue, the oximetry measurements are completely non-invasive and can be performed repeatedly for longer period of times (days, weeks and even years) [8].

We have focused on the development of formulations that could be applied topically on peripheral tumors to enhance tumor oxygenation. We report the effect of the formulations prepared by using a vasoactive compound, Benzyl Nicotinate (BN), on tissue pO_2 of subcutaneously grown RIF-1 tumors using EPR oximetry. The BN was incorporated in two different delivery systems (i) a hydrogel formulation and (ii) a water-in-oil microemulsion, and the changes in tumor pO_2 were followed with repeated applications of the BN formulations for five consecutive days.

2. MATERIALS AND METHODS

2.1. Animals and tumor models

All animal procedures were conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Dartmouth Medical School. The radiation-induced fibrosarcoma (RIF-1) cells were provided by Dr. J. B. Mitchell, National Cancer Institute. The cells were cultured in RPMI 1640 medium supplemented with 10 % FBS, and antibiotics. The procedure for tumor inoculation in female C3H mice (Charles River Laboratories, MA), and the methods to prepare the BN formulations have been reported previously [4, 10, 11].

2.2. Experiment design

The mice were divided into 4 groups: (i) BN-Hydrogel (n = 7) (ii) Hydrogel alone (n = 7) (iii) BN in the microemulsion (BN-ME) (n = 8) and (iv) the microemulsion (ME) alone (n = 5). The mice were anesthetized with 1.5 % isoflurane and 100% oxygen delivered through a nose cone, and a baseline (pre-treatment) tumor pO_2 was measured for 25 min using EPR oximetry described below. After baseline pO_2 measurements, a 0.2 ml of the formulation was gently applied on the tumor for 1 min without moving the mouse from the spectrometer, and the pO_2 measurements were continued for 60 min to determine the effect of the BN formulations on tumor pO_2 . The experiments were repeated for five consecutive days. In the control group, the formulations without BN (i.e. hydrogel alone or microemulsion alone) were applied and the same protocol was followed.

2.3. EPR Oximetry

EPR oximetry was performed with an L-band (1.2 GHz) EPR spectrometer equipped with an external loop resonator specifically designed for *in vivo* experiments [8, 9]. The lithium phthalocyanine (LiPc) crystals used for pO_2 measurements were synthesized in our laboratory; its physicochemical properties and use for measuring tissue pO_2 have been described previously [12]. Once the tumors reached a volume of 70 - 90 mm³, the LiPc (40 µg) crystals were loaded in 25 gauge needles and injected into the tumors at a depth of 1 - 2 mm from the surface. The EPR spectra obtained from these deposits reflect the average partial pressure of oxygen on the surface of the crystals. Tumor pO_2 was determined by measuring the peak-to-peak line-widths of the EPR spectra, which was then converted to pO_2 by using appropriate calibrations for the LiPc crystals used in this study.

For pO₂ measurements, the anesthetized mice (1.5% Isoflurane, 100% O₂) were placed between the poles of the EPR magnet and the resonator loop was placed gently over the tumor. The spectrometer parameters were: incident microwave power of 4 - 6 mW; modulation frequency 27 kHz; magnetic field 430 G; scan time 10 sec (usually 6 scans were averaged to increase signal to noise ratio), and modulation amplitude not exceeding one third of the peak-to-peak line-width (typical 0.02 - 0.05 G). During EPR measurements, the temperature of the animals was monitored using a rectal probe and maintained at 37 ± 0.5°C using a thermostatically controlled heated pad and a flow of warm air.

2.4. Data Analysis

The baseline tumor pO₂ was averaged for 25 min (shown at time 0) and the pO₂ data obtained after topical application of the formulations were averaged for 10 min each to assess temporal changes in tumor pO₂. A paired t-test was used to determine the statistical significance of the changes in pO₂ within the group. The tests were two-sided, and a change with a p value < 0.05 was considered statistically significant. All data are expressed as mean ± SE; n is the number of animals in each group.

3. RESULTS

3.1. Effect of the BN in hydrogel formulation on RIF-1 tumor pO₂

The RIF-1 tumors were hypoxic with a baseline pO₂ of 9.3 ± 0.8 mmHg and tumor volume of 71 ± 6 mm³ on day 1, Figure 1. The tumor pO₂ increased significantly to 11 ± 2 mmHg at 30 min after topical application of BN in hydrogel, and the pO₂ remained at a significantly higher level during 60 min of measurements. Experiments repeated on day 2 led to a significant increase in pO₂ at 20 - 30 min only. Similar results were obtained on days 3 - 5, but the magnitude and the time of a significant increase in tumor pO₂ declined over days. The baseline tumor pO₂ in the hydrogel alone group was 9.5 ± 0.8 mmHg, and no significant increase in tumor pO₂ was observed after topical application of hydrogel alone, Figure 2.

3.2. Effect of the BN in microemulsion formulation on RIF-1 tumor pO₂

The RIF-1 tumors were hypoxic with a baseline pO₂ of 8.8 ± 0.3 mmHg and tumor volume of 80 ± 8 mm³ on day 1. The tumor pO₂ increased significantly within 10 min of topical application of the BN formulation, Figure 3. A maximal increase in tumor pO₂ to 18.5 ± 0.9 mmHg was observed at 20 min, and the tumors remained at a significantly oxygenated level during 60 min of measurements. Experiments repeated on day 2 also resulted in a significant increase in tumor pO₂, which declined after 30 min of topical application. Similar results were obtained on day 3 and day 4, but no significant increase in tumor pO₂ was observed on day 5. The baseline tumor pO₂ of the control group was 8.2 ± 0.7 mmHg on day 1, and no significant increase was observed after the topical application of microemulsion alone during 5 days of experiments, Figure 4.

4. DISCUSSION

The goal of this study was to enhance tissue oxygenation of peripheral tumors by an innovative approach, i.e. by topical application of a vasoactive compound onto the tumors. The topical application of drugs has many potential advantages including high patient compliance, sustained and controlled delivery over time as desired and less toxicity as compared to systemic treatments. Benzyl Nicotinate was selected as a vasoactive drug, on the basis of its lipophilicity that makes it a good candidate for skin penetration. Enzymatic hydrolysis of BN results in nicotinic acid, which increases cutaneous blood flow locally and as a consequence increases tissue oxygenation [10, 11].

The results indicate a significant increase in tumor pO₂ following BN application in both of the formulations tested. The magnitude and the time of maximal increase in tumor pO₂ declined over days, and by day 5, a little or no change was observed. In the control groups (formulations without BN), no increase in tumor pO₂ was observed as compared to the baseline pO₂, which confirms that the increase in tumor pO₂ is likely due to the vasodilating effect of Benzyl Nicotinate. However, a variation in the baseline tumor pO₂ over days was observed in these experiments. A further optimization of these formulations is necessary to achieve an optimal and consistent vasodilating effect during repeated applications. Nevertheless, the increase in tumor pO₂ observed in this study potentially could be used to enhance the radiotherapeutic outcome of peripheral tumors, if irradiated at the time of a significant increase in tumor pO₂ observed after the topical application of BN formulations.

In conclusion, these results provide temporal information on the effectiveness of BN formulations in enhancing tumor oxygenation and the potential of EPR oximetry to monitor these changes repeatedly during such treatments. Further modifications are being carried out to optimize these formulations for their potential application with radiotherapy to improve radiotherapeutic outcome.

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Figure 1.

Tissue pO₂ of RIF-1 tumors measured before (time = 0 min) and after the topical applications of the BN formulation in hydrogel. ◇ day 1; □ day 2; △ day 3; ○ day 4; * day 5. *p < 0.05 compared with baseline pO₂, mean + SE, n = 7.

Figure 2. Tissue pO₂ of RIF-1 tumors measured before (time = 0 min) and after the topical applications of the hydrogel alone (control group). ◇ day 1; □ day 2; △ day 3; ○ day 4; * day 5. *p < 0.05 compared with baseline pO₂, mean + SE, n = 7.

Figure 3.

Tissue pO₂ of RIF-1 tumors measured before (time = 0 min) and after the topical applications of the BN formulation in microemulsion. ◇ day 1; □ day 2; △ day 3; ○ day 4; * day 5. *p < 0.05 compared with baseline pO₂, mean + SE, n = 8.

Figure 4.

Tissue pO₂ of RIF-1 tumors measured before (time = 0 min) and after the topical applications of the microemulsion alone (control group). ◇ day 1; □ day 2; △ day 3; ○ day 4; * day 5. *p < 0.05 compared with baseline pO₂, mean + SE, n = 5.