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# Oxygenation and blood flow in tumors treated with hydralazine: Evaluation with a novel luminescence-based fiber-optic sensor

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**Abstract.** Two optical methods were used simultaneously to evaluate the effect of a vasoactive drug hydralazine on oxygenation and blood flow in SA-1 tumors in A/J mice. A novel luminescence-based optical sensor (OxyLite instrument, Oxford Optronix, UK) was used to monitor partial pressure of oxygen ($pO_2$) in tumors. Laser Doppler flowmetry was used to assess relative blood perfusion in tumors. Measurements were performed continuously on anesthetized mice before and after i.v. injection of hydralazine at a dose of 2.5 mg/kg. Hydralazine reduced $pO_2$ on average by 80% and blood flow by 50% within ten minutes after injection. Our results indicate that hydralazine could be used to improve the antitumor effectiveness of hypoxic cell-specific therapies. We demonstrated that both optical methods can be used successfully to detect changes in blood perfusion and oxygenation in tumors after blood flow-modifying treatments. Some aspects of $pO_2$ measurement with the new luminescence-based method require further investigation.

Keywords: Experimental tumors, hydralazine, oxygenation, blood flow, OxyLite luminescence-based fiber-optic sensor, laser Doppler flowmetry

## 1. Introduction

Abundant evidence has accumulated over many years that the oxygenation status in both experimental and clinical tumors can influence the response of these tumors to various therapies [9,12,31]. These therapies include radiotherapy, hyperthermia, oxygen-dependent chemotherapy, photodynamic therapy and cell-mediated immunotherapy. The oxygenation status and hypoxia in particular is also important for the development of malignant growth and for progression and outcome of the disease once it has developed [31]. It is therefore clear that the ability to measure oxygenation status accurately in individual tumors would be very valuable for selection of appropriate therapies and for prediction of the treatment outcome.

Many different methods have been developed to measure different physiological parameters related to tumor oxygenation or to tumor blood flow [10,22]. The task of measuring tumor oxygenation and blood flow is by no means an easy one. Since tumors are in general characterized by poor oxygenation in comparison to normal tissues [9,22,31], it is important that the methods we use can measure accurately

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very low oxygen levels. Due to extreme intratumoral heterogeneity in oxygenation and blood flow encountered in many experimental and clinical tumors, it is desirable that the methods of choice enable evaluation of oxygenation and blood flow in different regions within the same tumor. And finally, it can be extremely useful if the measurement method can be applied continuously. In this way the changes in oxygenation at one location can be monitored. This would be useful for at least two reasons. First, the effectiveness of treatment procedures targeting the tumor blood flow or oxygenation in an individual tumor could be evaluated. Second, the susceptibility of an individual tumor to oxygenation- and blood flow-dependent treatment could be evaluated before the treatment in question is applied.

Two optical methods, which we used in the present study, fulfill some of the requirements mentioned earlier in this text. One of them, the time-resolved luminescence-based optical oximetry, presents a novel approach in measurement of oxygen partial pressure in tissue and is an alternative to the well-established polarographic needle oximetry technique [7,8]. This method has only recently become commercially available as the OxyLite instrument produced by Oxford Optronix. Its major advantage over standard polarographic method is absence of oxygen consumption by the sensor. The other optical method used in our study, the laser Doppler flowmetry, is not a new technique but it has not been used extensively for measurements in tumors.

Another physiological condition typically encountered in solid tumors is elevated hydrostatic pressure in extracellular space or interstitial fluid pressure – IFP [2,13,17]. High IFP in tumors presents an obstacle for delivery of drug molecules to tumor cells. Elevated IFP arises among other reasons due to absence of lymphatic vessels and due to abnormal blood vasculature in tumors. Evidence exists that IFP inside tumors is determined by the balance between fluid flow from the vasculature into tumor interstitium and from the interstitium into the surrounding normal tissue [2]. It has also been speculated that increased IFP in tumors may lead to vascular collapse of some capillaries and thus to development of hypoxia, but this has not been confirmed yet [17]. There is however some evidence that high IFP in tumors is in general associated with poor blood flow and oxygenation. High IFP in human cervix cancer, for example, was found to be associated with low oxygenation and resistance to radiotherapy [17]. But other authors found no general correlation between IFP and oxygen partial pressure in solid tumors [3]. Therefore it is not clear whether elevated IFP causes and contributes to tumor hypoxia through changes in blood flow, or elevated IFP is simply associated with hypoxia in some tumors.

One of possible approaches in tumor treatment is to reduce the resistance of tumors to standard therapies through modification of physiological constraints that cause the resistance to therapy. Such physiological constraints are elevated IFP and poor oxygenation and blood flow in tumors. In some studies, including our own, it was found that hydralazine, a long-acting arteriolar vasodilator used in humans for treatment of hypertension, can effectively reduce IFP in experimental tumors in mice [21, 32]. It is also known that application of hydralazine effectively reduces blood flow in tumors at high doses [11,16,18,32], but may also increase the tumor blood flow at low doses [11,18]. The first effect, decreased IFP due to hydralazine, might contribute to improved oxygenation of tumors if the relationship between high IFP and low oxygenation exists [17]. The second effect however, the decreased blood flow due to hydralazine at high doses, should further decrease oxygenation in tumors.

The main goal of the present study was therefore to evaluate the effect of hydralazine on blood flow and oxygenation simultaneously in SA-1 fibrosarcoma tumors in A/J mice. In addition, the purpose of the study was to evaluate the usefulness of the novel optical method for continuous measurement of tumor oxygenation and for detection of short term oxygenation changes.

## 2. Materials and methods

### *2.1. Animals and tumors*

The female A/J mice used in our study were purchased from Rudjer Boškovič Institute, Zagreb, Croatia. The mice were 10 to 14 weeks old at the beginning of experiments. They were kept in standard animal colonies at 22°C and were fed and watered ad libitum. The experimental tumor line used was SA-1 fibrosarcoma (The Jackson Laboratory, Bar Harbor, USA). Tumor cells for inoculation of solid tumors were obtained from the ascitic form of SA-1 tumor in A/J mice. Approximately $5 \times 10^5$ of viable cells were resuspended in 0.1 ml NaCl solution (0.9%) and transplanted under the skin. Solid subcutaneous tumors were grown dorsolaterally in the right flank of mice. Experiments were performed 7 to 10 days after transplantation when tumors reached the size of approximately 100 $mm^3$. The size of the tumors was calculated using the ellipsoid formula as $V = \pi abc/6$ where $a$, $b$, and $c$ are three mutually perpendicular tumor diameters measured by a vernier caliper.

At the end of the experiments the animals were euthanized. First the delivery of $O_2$ was eliminated from anesthetic gas mixture while maintaining the flow of nitrous oxide and isoflurane. For details on anesthesia see below. Mice stopped breathing within two minutes after this procedure after which they were killed by cervical dislocation. Experimentation on mice was conducted in accordance with the legislation concerning animal care and protection and was approved by the national governing authorities.

### *2.2. Anesthesia*

All invasive experimental procedures and measurements were conducted on anesthetized mice; first, to eliminate pain and discomfort in mice; and second, to minimize movements of otherwise non-restrained mice during long-lasting measurements. Inhalation anesthetic isoflurane (Flurane-Isoflurane, Abbot Labs, UK) was used for induction and maintenance of anesthesia. The gas mixture of oxygen $O_2$ and nitrous oxide $N_2O$ (flow of each 0.6 l/min) containing isoflurane at 1.7% concentration was delivered to the mouse via a miniature face mask. While anesthetized, the animals were kept on an automatically regulated heating pad to prevent hypothermia. Rectal temperature was kept as close as possible to 37 °C (variations of up to 0.5°C were possible during single measurements) with the contact temperature of the heating pad never exceeding 39°C. The ambient temperature during the experiments was 22–24°C.

### *2.3. Hydralazine treatment*

Hydralazine (HYZ) was injected i.v. at a dose of 2.5 mg per kg of mouse weight. The solution for injection was prepared from powdered HYZ (Hydrazinophthalazine, Sigma Chemical Co., USA) by dissolving it in sterile physiological saline (0.9% NaCl) at concentration 1 mg of HYZ per 3 ml of saline. Mice were injected with 0.075 ml of this solution per 10 g of body weight thus resulting in the aforementioned dose of 2.5 mg/kg. Mice in the control group were injected with 0.075 ml of sterile physiological saline per 10 g of body weight.

### *2.4. Oxygenation and blood flow measurement*

#### *2.4.1. Oxygenation measurement*

We measured $pO_2$ with the OxyLite 2000 instrument (Oxford Optronix Ltd., Oxford, UK), a commercially available implementation of the time-resolved luminescence-based optical oximetry. This

instrument has two independent channels for measurement of $pO_2$ and temperature. The diameter of precalibrated optical probes is 230 $\mu$m. A thin wire thermocouple temperature sensor (diameter less than 100 $\mu$m) is attached to each $pO_2$ probe which allows the on-line temperature correction of $pO_2$ measurements. According to the producer, the response time of their $pO_2$ sensor is less than 10 seconds and the accuracy of measurement is 0.7 mmHg or $< \pm 10\%$ of the measured value, whichever is greater [19]. OxyLite 2000 enables continuous monitoring of $pO_2$ during long periods of time. The output signal of each of the two measurement channels is updated every two seconds. During a two seconds period, each sensor collects data for one second. When one sensor is active, the other one is idle. In our study the update mode on the instrument was set to "5 seconds". This means that the output value of each of the two measurement channels, which updated every two seconds, was calculated as a moving average value of 5 seconds of measurement collected over the last period of 10 seconds. All $pO_2$ values are expressed in millimeters of mercury (mmHg) because this unit is still predominantly used in clinical environment. One mmHg equals 133.332 Pa.

The principles of the time-resolved luminescence-based optical method are described in more detail elsewhere, e.g. [7,19], but briefly they can be summarized as follows. In this method, pulses of blue light emitted by a LED diode are carried via an optical fiber to ruthenium chloride luminophore which is incorporated into a silicone rubber immobilized at the probe tip. The probe tip must be placed inside the tissue where oxygenation is to be measured. The incident light pulses induce pulsatile fluorescence of ruthenium molecules at a wavelength longer than that of the incident blue light. This fluorescence decays (is being quenched) in time because of collisions between the oxygen and the ruthenium molecules. The life-time of the excited fluorescence is inversely proportional to oxygen tension (oxygen partial pressure; $pO_2$) in immediate vicinity of the probe tip and can thus be measured. Using the so-called Stern-Volmer relation, the $pO_2$ can be calculated by comparing the measured life-time of the fluoresced light to that obtained in absence of oxygen. In addition to tissue $pO_2$, tissue temperature also affects the life-time of the excited ruthenium fluorescence. The higher the temperature, the shorter the life-time. This effect can introduce errors into calculations of $pO_2$ unless the temperature is also measured at the same location as $pO_2$ and used for compensation in calculations. Sensors of the luminescence-based method do not consume oxygen. Therefore they can be left in one place in tissue to monitor $pO_2$ changes over longer periods. This is supposedly the major advantage of this new method over the well established polarographic oximetry.

*2.4.2. Blood flow measurement*

Relative blood flow or blood perfusion was monitored using OxyFlo 2000 laser Doppler instrument (Oxford Optronix Ltd., Oxford, UK). This LDF instrument has two independent channels for measurement of blood perfusion and uses two semiconductor diodes to produce laser light with the wavelength of 830 nm. In principle the LDF technique can be used entirely noninvasively if the probe (a pair of transmitting and receiving optical fibers) is attached to the surface of skin. But in this case the resulting measurement would reflect only skin blood perfusion. In order to assess perfusion inside the tumor we used thin invasive probes (diameter 200 $\mu$m) which were inserted into the tissue. According to the producer, the response time of their LDF instrument is 0.05 seconds which enables continuous monitoring of very rapid changes in blood flow.

Laser Doppler flowmetry (LDF) is an optical method used to monitor local microvascular blood flow in tissue. Extensive literature exists on both theory and application of LDF, e.g. [20,26]. Briefly, the underlying physical principle is as follows. When a tissue is illuminated by coherent laser light, light interacts with tissue structures. Among other types of interaction, scattering of light is the most important

for LDF measurements. When photons are scattered on static structures in tissue, their wavelength does not change. But when photons are scattered on moving structures in tissue, their wavelength slightly changes. This is the so-called Doppler shift effect which can be measured. If other movements in tissue are negligible, the predominant remaining moving structures in tissue are red blood cells. Their movement which results from blood flow, can then be detected by means of Doppler shift effect. The output signal of LDF is therefore proportional to the blood cell perfusion which in turn is defined as the number of blood cells moving in the tissue sampling volume multiplied by the mean velocity of these cells. Unfortunately the constant of proportion between actual perfusion and the detected LDF signal is different for different locations even within the same tissue. This means that all LDF measurements are intrinsically of relative nature and are used mainly to detect changes in blood flow as a function of time. LDF measurements are quantified in arbitrary blood perfusion units (BPU).

#### *2.4.3. Data acquisition*

Analog outputs of both instruments, OxyLite and OxyFlo, were connected to OxyData data acquisition unit (Oxford Optronix Ltd., Oxford, UK) which enabled data storage to a PC via a SCSI connector. Chart 3.4 software (ADInstruments, Castle Hill, Australia) supplied by Oxford Optronix was used for data collection. All signals were sampled and stored for further analysis at frequency of 20 Hz. The purpose of this relatively high sampling frequency was to gather LDF data at high enough time resolution for future frequency analysis.

### *2.5. Measurement protocol*

Anesthesia of a mouse was started in induction chamber at 3% concentration of isoflurane. About one minute after induction of anesthesia the mouse was transferred to the heating pad. In Fig. 1 the experimental setup is presented. The mouse was placed in prone position and no physical restriction was used. The delivery of anesthetic gas was secured via a miniature face mask. Concentration of isoflurane was reduced to 1.7% which provided stable anesthesia. Special rectal and surface temperature probes were then attached for the control of the core temperature of the mouse and of the heating surface temperature respectively. Approximately four minutes after induction of anesthesia two $pO_2$ and two LDF probes were inserted into tumor. A small superficial incision in the skin was first made for each probe with a 0.9 mm hypodermic needle. The probes were then advanced through the incisions few millimeters into the tumor and then slightly (for approximately 0.5 mm) withdrawn in order to minimize the pressure of the probe tip on the surrounding tissue. All four probes were inserted into tumor approximately in parallel from the cranial side of the tumor. Exact positioning of the probe tip inside the tumor was not possible but in general one $pO_2$ probes was inserted in the peripheral region of the tumor and the other $pO_2$ probe was inserted in central region of the tumor. LDF probes were inserted at an 1–2 mm distance from the $pO_2$ probes. Position of all four probes was secured to certain extent by taping them to the heating pad surface. After the probes had been secured in the tissue, data recording was started. This normally occurred about five minutes after the start of anesthesia. Only in a few cases we started data recording before inserting the probes. Special care was taken throughout the measurements not to move the probes or the mouse in order to minimize the movement artifacts in recorded signals. This was particularly important for the highly sensitive LDF method.

An average measurement lasted between one and a half hour and two hours. The treatment (injection of either HYZ or physiological saline) was performed only after the recorded signals had been stable for at least ten minutes. Normally the stability of signals was reached between 30 and 60 minutes after the start of anesthesia. Recording of measurements was continued for at least 40 minutes after the injection.

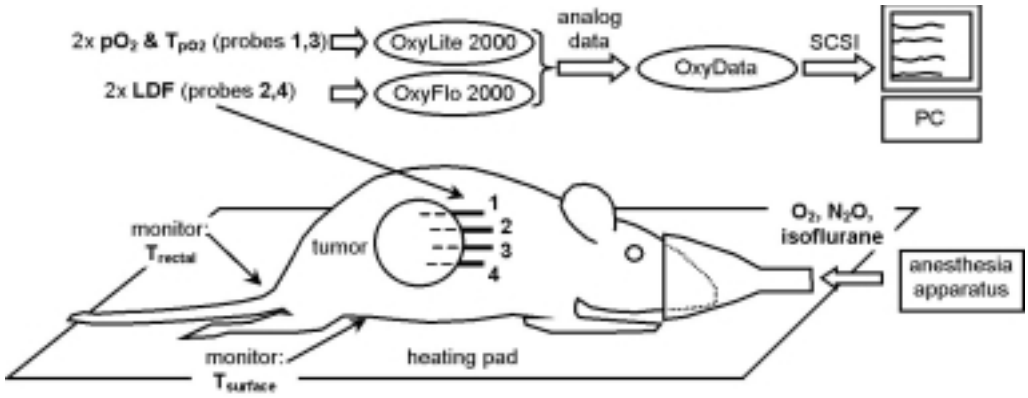


Fig. 1. Experimental setup for blood perfusion (LDF) and oxygenation ($pO_2$) monitoring in tumors growing subcutaneously in the right flank of mice.

### *2.6. Statistical methods*

All averaged results are presented as median values with 25th percentile and 75th percentile error bars. Mean values with standard deviation bars were not used because the normality test was not always fulfilled. Statistical significance of the effect of treatment in tumors were evaluated with Wilcoxon signed rank test. Mann-Whitney rank sum test was used to evaluate statistical significance of differences between tumors treated with HYZ and control tumors. The difference between groups was considered statistically significant for the values of $p < 0.05$ but we also provide explicit values of $p$ for each particular case in the Results section. All statistical calculations were performed with SigmaStat 2.03 software (SPSS Inc., USA).

## 3. Results

In all mice included in the experiments, $pO_2$ and blood perfusion were measured at two locations each. This was possible because both instruments, OxyLite 2000 and OxyFlo 2000 have two measurement channels. In some mice one pair of probes (one $pO_2$ and one LDF probe) was inserted into tumor and the other pair was inserted under the skin into normal tissue either in the right leg or in the right flank in vicinity of the tumor. In majority of mice however, all four probes (two $pO_2$ and two LDF) were inserted into tumor because we have found out during the experiments that relatively high $pO_2$ in normal tissue frequently rendered measurements of $pO_2$ unstable. All the results presented in this section were obtained from tumors.

Measurement of $pO_2$ and blood perfusion was started immediately after all four probes were inserted into tissue or, in a few cases, just before the probes were inserted. All this normally occurred about five minutes after the start of anesthesia. The measurement was then maintained for approximately two hours. The examples of a pair of measurements ($pO_2$ and LDF) from a control tumor and from two HYZ-treated tumors are shown in Figs 2, 3, and 4.

### *3.1. General observations*

#### *3.1.1. Oxygenation*

The first important feature present in all $pO_2$ measurements in tumors was that immediately after insertion of the probe the value of $pO_2$ rapidly fell to zero or close to zero. This zero $pO_2$ value can be

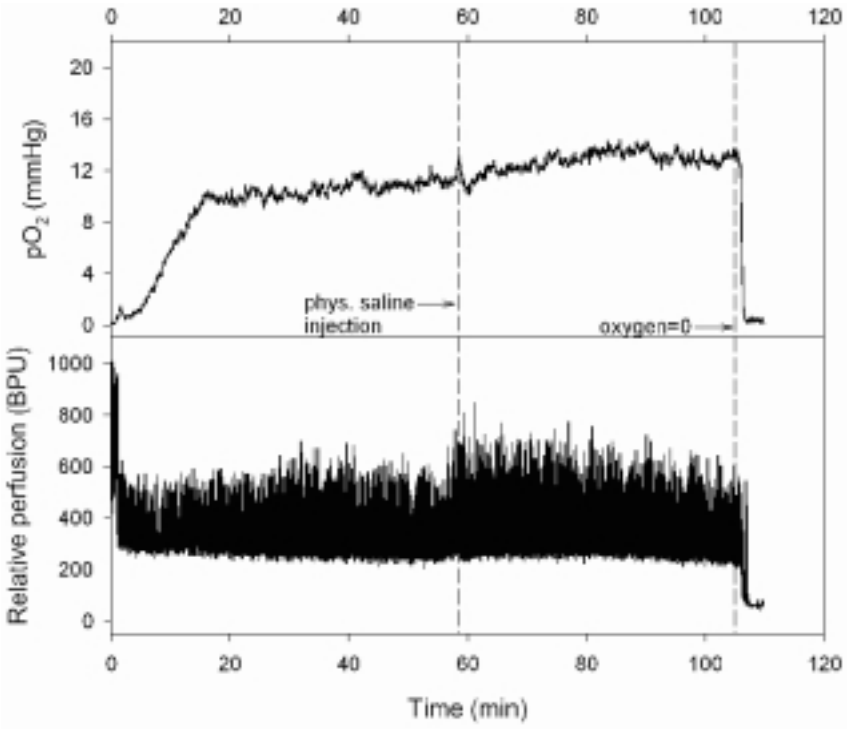


Fig. 2. Partial pressure of oxygen (top) and relative blood perfusion (bottom) in control tumor sampled at frequency 1 Hz (type I $pO_2$ measurement). The two vertical lines show when physiological saline was injected and when oxygen supply to inhalation gas was terminated.

observed in top graphs of Figs 2, 3, and 4. The initial rapid decrease cannot be observed in these figures because the data recording was started only after the probes had been inserted. But in all cases where data recording was started before the probes were inserted, this rapid decrease occurred within the first minute after insertion (not shown). Two distinct types of $pO_2$ measurements were found in our study. In about one third of all $pO_2$ measurements performed, $pO_2$ in tumors remained at zero level for the entire period of observation. This can be observed in Fig. 4 and we call this a "type II" $pO_2$ measurement. In such cases no effect of hydralazine or of any other procedure on $pO_2$ could be measured. In the remaining two thirds of measurements, we will call these "type I" measurements, $pO_2$ in tumors started to increase after a certain period (see Figs 2 and 3). The period of zero or close to zero $pO_2$ value in type I measurements was sometimes very short (one minute or less as in Figs 2 and 3) or as long as 20 minutes. But in most cases this phase of the initial "zero level" $pO_2$ lasted between three and five minutes. In all type I measurements the $pO_2$ then started to increase. The increase phase lasted between 15 and 20 minutes on average. After this increase phase a plateau phase was reached. This can be seen in both examples in Figs 2 and 3. In this plateau phase the value of $pO_2$ stabilized and remained mostly unchanged for the entire remaining period of observation unless a procedure such as injection of HYZ was performed. In most cases $pO_2$ continued to increase very slowly even during this plateau phase but this increasing was much slower than the increasing in the previous phase (see Fig. 2).

The described behavior of $pO_2$ signal in time (type I) was observed in all measurements in tumors except in cases where $pO_2$ remained at zero or close to zero level for the entire period of observation (type II). In order to obtain the representative $pO_2$ value at rest before treatment for each measurement

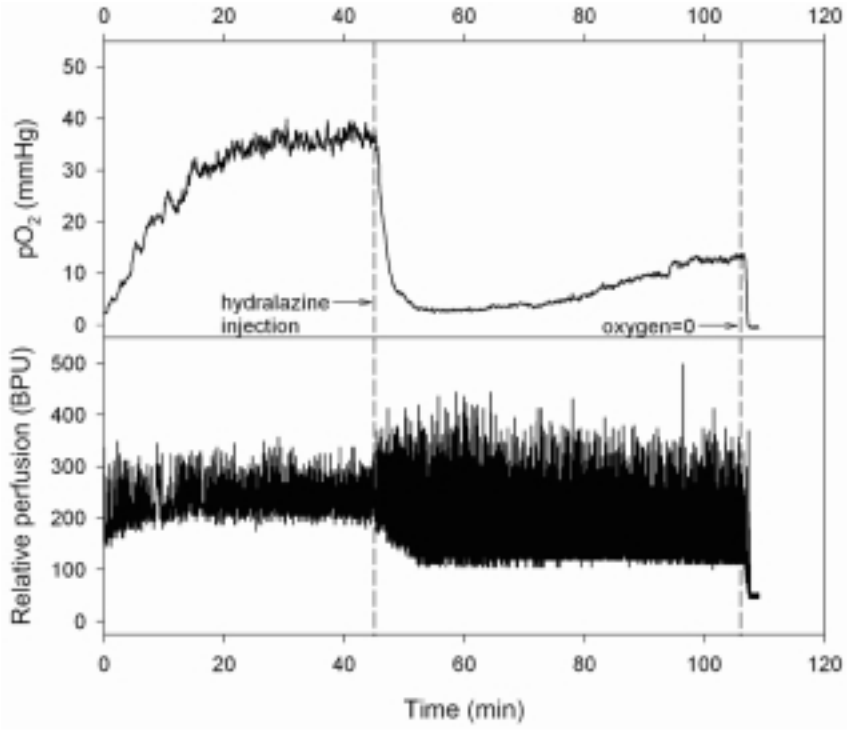


Fig. 3. Partial pressure of oxygen (top) and relative blood perfusion (bottom) in hydralazine-treated tumor sampled at frequency 1 Hz (type I $pO_2$ measurement). The two vertical lines show when hydralazine solution (2.5 mg/kg) was injected and when oxygen supply to inhalation gas was terminated.

location, we averaged the raw $pO_2$ signal in the plateau phase over the last five minutes just before the injection of either physiological saline or HYZ. It is important to note that these averaged $pO_2$ values varied extremely between tumors and also from one location to another within the same tumor. All tumor $pO_2$ values at rest thus obtained from the control tumors, the HYZ-treated tumors and from some tumors, which were not directly included in the hydralazine study, were pooled together and are presented in a histogram in Fig. 5. It follows from this histogram that some recorded $pO_2$ values at rest (some of type II measurements) were even slightly negative in the range between $-0.7$ and 0.0 mmHg. Even though negative $pO_2$ has no meaning we did not correct these values because they were within the accuracy of OxyLite instrument ($\pm$ 0.7 mmHg [19]). The median and the mean $pO_2$ value of 40 measurements from 28 tumors were 10.3 and 17.4 mmHg respectively. Fraction of $pO_2$ values below 2.5, 5.0, and 10.0 mmHg were 40%, 45%, and 50% respectively.

### *3.1.2. Blood flow*

Contrary to the $pO_2$ signals, the blood perfusion LDF signals became stable much sooner in the course of measurement. This can be verified by comparing the top and the bottom graphs in Figs 2 and 3. Blood perfusion signal never started off with a zero level as $pO_2$ signals did. LDF signals were generally more stable than $pO_2$ signals during the whole period of observation. Another important feature of perfusion measurements is that we never encountered any location within tumors with zero blood perfusion. This again is contrary to the $pO_2$ measurements where one third of all measurements resulted in zero or close to zero $pO_2$ throughout the period of observation as we have already mentioned (type II measurements).

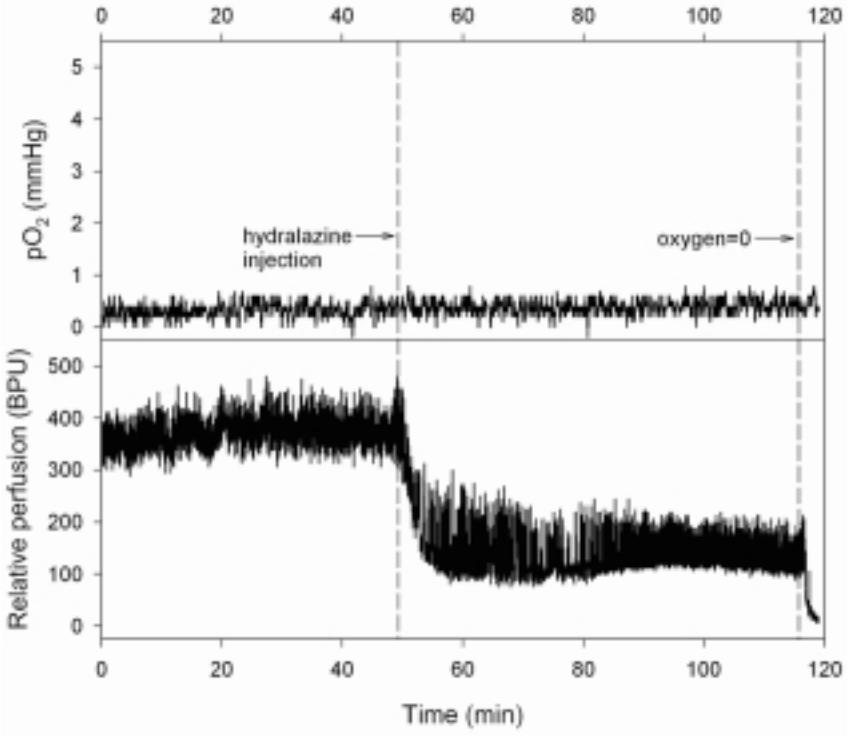


Fig. 4. Partial pressure of oxygen (top) and relative blood perfusion (bottom) in hydralazine-treated tumor sampled at frequency 1 Hz (type II $pO_2$ measurement). The two vertical lines show when hydralazine solution (2.5 mg/kg) was injected and when oxygen supply to inhalation gas was terminated.

Due to the nature of LDF technique, the blood perfusion measurements were much more sensitive than $pO_2$ measurements to any kind of movement of the probes. The "smeared" blood perfusion signals in Figs 2, 3, and 4 include a movement artifact which is a result of quasi-periodic movement of tumor caused by breathing of the mice. A 30 seconds long segment of blood perfusion signal from the bottom graph in Fig. 2 is shown in more detail in Fig. 6. Each spike in Fig. 6 corresponds to the moment of maximum inhalation of the mouse. The flat "valleys" in between two neighbor spikes correspond to maximum exhalation state. The actual level of relative blood perfusion is hidden in the "valleys" between the spikes. It follows that the actual blood perfusion is represented by the bottom of the "smeared" blood perfusion signals in Figs 2, 3, and 4. The periodic change in blood perfusion signal caused by movement of tumor during inhalation/exhalation could not be avoided due to the location of tumors on thorax. The amplitude of this quasi-periodic component in LDF signal was in many cases bigger than the DC component of the signal. The amplitude of the quasi-periodic change varied a lot between measurement locations and depended on individual location of the tumor on mouse's flank and on the position of LDF probe relative to the direction of tumor movement caused by respiration. The amplitude of the quasi-periodic change in perfusion signal also depended on the depth of respiration. When for example breathing became deeper or more jerky, this reflected in increased amplitude of perfusion signal change.

Blood perfusion measured by LDF was quantified in arbitrary blood perfusion units. This is one of the reasons why LDF results in general do not allow direct comparison of perfusion values at rest obtained from different locations. However, relative changes in perfusion can be compared.

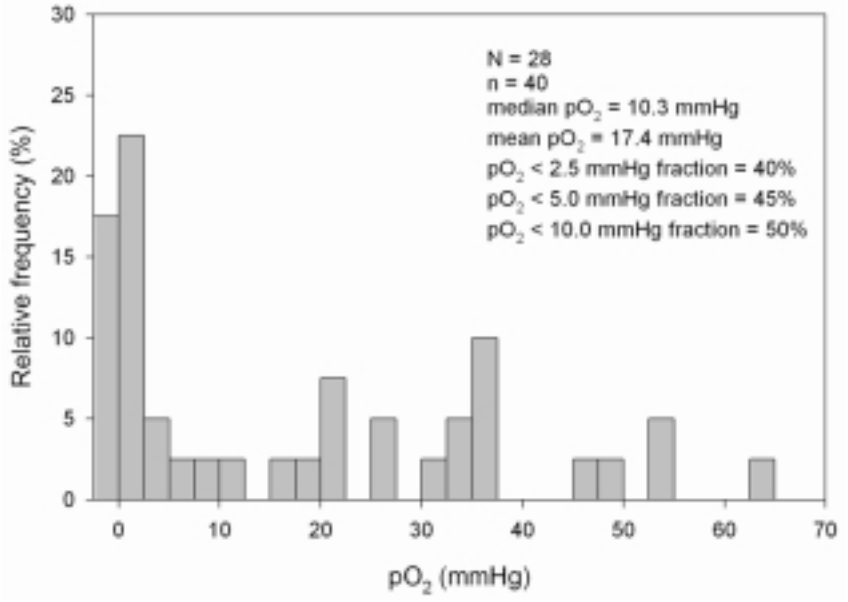


Fig. 5. Distribution of tumor $pO_2$ values at rest. Measurements from all tumors are pooled together ($N$ = number of tumors; $n$ = number of measurements).

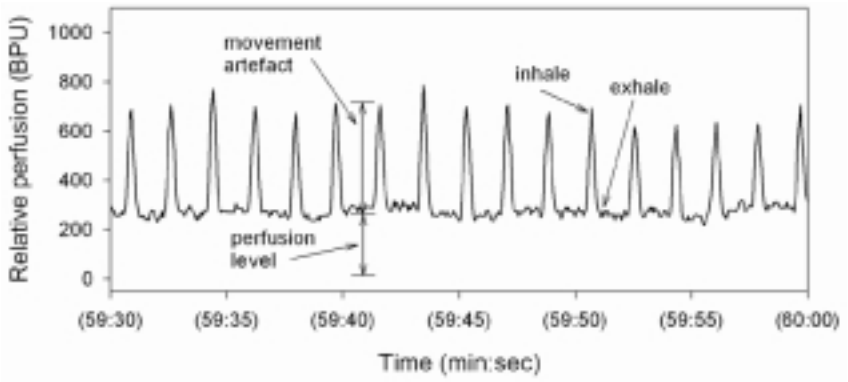


Fig. 6. A close-up of relative blood perfusion trace from the bottom graph in Fig. 2 (control tumor) sampled at frequency 20 Hz. The last 30 seconds of the 60th minute of recording are shown. The movement artifact caused by quasi-periodic respiration of the mouse is shown.

### *3.2. Effect of hydralazine*

Within one minute after the injection of HYZ both $pO_2$ and blood perfusion in tumor started to decrease rapidly as can be seen in the example in Fig. 3 for $pO_2$ and in Figs 3 and 4 for blood perfusion. In type I cases the $pO_2$ decreased faster than the perfusion and in most cases the $pO_2$ signal reached the lowest level before the perfusion signal did. Both signals then remained decreased for a certain period of approximately 20 minutes and then started to recover slowly. However, during the period of observation neither $pO_2$ nor blood perfusion returned to the pretreatment level. Values of both variables remained significantly lower than before treatment. LDF signal in Fig. 3 also shows that the depth of respiration increased significantly after injection of HYZ. This occurred in most HYZ-treated mice. This

Table 1
$pO_2$ and blood perfusion at four different intervals following the injection of hydralazine at a dose 2.5 mg/kg. Median values and 25th and 75th percentiles are shown. Wilcoxon signed rank test (value of $p$) was used to calculate statistical significance between $pO_2$ and blood perfusion at 10, 20, 30, and 40 minutes after injection and the pre-injection value. $N$: number of mice; $n$: number of measurements.

| Hydralazine | $pO_2$ (mmHg) $N = 12;\ n = 17$ | | | | Blood perfusion (BPU) $N = 12;\ n = 17$ | | | |
|---|---|---|---|---|---|---|---|---|
| | median | 25% | 75% | $p$ | median | 25% | 75% | $p$ |
| pre-injection | 17.7 | 2.9 | 36.6 | – | 245 | 120 | 431 | – |
| 10 min | 2.1 | 0.2 | 7.3 | $< 0.001$ | 138 | 85 | 267 | $< 0.001$ |
| 20 min | 2.4 | 0.2 | 4.4 | $< 0.001$ | 125 | 64 | 253 | $< 0.001$ |
| 30 min | 3.0 | 0.4 | 6.7 | $< 0.001$ | 150 | 78 | 268 | $< 0.001$ |
| 40 min | 4.6 | 0.4 | 11.0 | $< 0.001$ | 138 | 88 | 331 | $< 0.001$ |

Table 2
$pO_2$ and blood perfusion at four different intervals following the injection of physiological saline (control). Median values and 25th and 75th percentiles are shown. Wilcoxon signed rank test (value of $p$) was used to calculate statistical significance between $pO_2$ and blood perfusion at 10, 20, 30, and 40 minutes after injection and the pre-injection value. $N$: number of mice; $n$: number of measurements.

| Control | $pO_2$ (mmHg) $N = 5;\ n = 7$ | | | | Blood perfusion (BPU) $N = 6;\ n = 8$ | | | |
|---|---|---|---|---|---|---|---|---|
| | median | 25% | 75% | $p$ | median | 25% | 75% | $p$ |
| pre-injection | 22.2 | 13.6 | 32.7 | – | 540 | 341 | 790 | – |
| 10 min | 21.5 | 14.5 | 30.1 | 0.938 | 538 | 325 | 806 | 0.938 |
| 20 min | 26.8 | 15.2 | 32.9 | 0.219 | 540 | 332 | 815 | 0.219 |
| 30 min | 27.2 | 15.5 | 38.1 | 0.078 | 550 | 331 | 778 | 0.938 |
| 40 min | 27.3 | 14.9 | 36.8 | 0.109 | 545 | 338 | 766 | 0.688 |

effect is reflected in more than doubled width of the "smear" in this LDF signal. At the same time the respiration rate decreased (data not shown). In most cases the increase in depth of respiration and the decrease in respiration rate remained visible during the whole remaining period of observation. All changes described here were observed in all HYZ-treated tumors except for the "all-zero" type II $pO_2$ measurements where the value of $pO_2$ was zero for the whole period of observation. In Table 1 we summarize the effect of HYZ on $pO_2$ and blood perfusion in tumors including the "all-zero" type II $pO_2$ measurements. It clearly follows from these data that hydralazine induced statistically highly significant reduction of tumor blood flow and oxygenation. Values of $p$ of Wilcoxon signed rank test comparing the individual values of $pO_2$ and blood flow before injection with these values at intervals 10, 20, 30, and 40 minutes after injection were less than 0.001.

In comparison to the HYZ-treated tumors, injection of physiological saline did not produce significant changes neither in $pO_2$ nor in blood perfusion. In some cases there was a transient change in $pO_2$ and an increased depth of respiration detected immediately after injection of the saline. An example of this can be seen in Fig. 2. The results obtained from all control tumors (including the "all-zero" type II $pO_2$ measurements) are summarized in Table 2. It follows clearly from these data that injection of physiological saline alone did not induce significant changes in tumor blood flow and oxygenation.

For the purpose of graphical comparison of the results of HYZ-treated and control tumors, we further processed the raw data. All individual values of $pO_2$ (except for the "all-zero" values) measured at 10, 20, 30, and 40 minutes after injection of HYZ (or physiological saline) were expressed as a percentage of the $pO_2$ value measured at the same location at rest before the injection. This means that all values

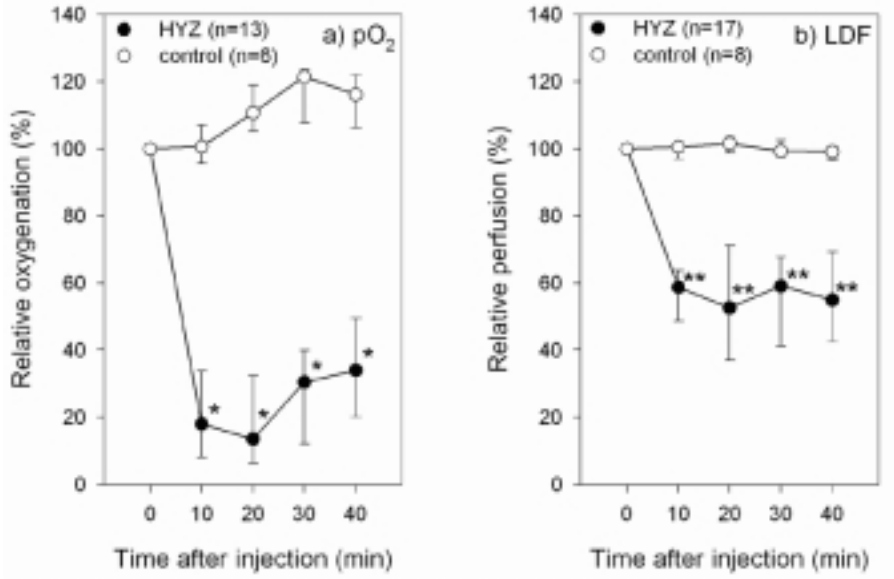


Fig. 7. Averaged relative oxygenation (a) and relative blood perfusion (b). Median values with 25th and 75th percentile error bars are shown. All individual absolute values of $pO_2$ and blood perfusion were normalized with respect to the corresponding absolute values before injection of hydralazine (HYZ) or physiological saline (control). Mann-Whitney rank sum test was used to evaluate statistical significance between HYZ and control group at 10, 20, 30, and 40 minutes after injection ($n$: number of samples; *: $p = 0.002$; **: $p < 0.001$).

of $pO_2$ after treatment were normalized with respect to the pretreatment value. The same procedure was also applied to the blood perfusion data: the values of blood perfusion measured at 10, 20, 30, and 40 minutes after injection were normalized with respect to the corresponding pretreatment value. The results thus obtained are presented in Fig. 7. Each data point in this figure corresponds to the median value. Error bars used are 25th percentile and 75th percentile. It again follows from these two figures that hydralazine produced significant reduction of tumor blood flow and oxygenation in comparison to control tumors.

## 4. Discussion

### *4.1. Effect of hydralazine on tumor blood flow and oxygenation*

The results of our study demonstrate that hydralazine at a dose of 2.5 mg/kg significantly reduced blood flow and oxygenation of SA-1 fibrosarcoma tumors in A/J mice. The effect was seen in all tumors. Blood perfusion measurements by LDF method showed that on average blood flow decreased in 10 to 20 minutes after injection of HYZ by approximately 50% from the pretreatment level (Fig. 7(b)). Even though it can not be seen from the averaged data in Fig. 7(b), some of tumors exhibited a partial reperfusion starting about 30 minutes after administration of HYZ (see recordings from individual tumors in Figs 3 and 4).

These result are in good agreement with the results obtained by other authors in various mouse tumor models. Trotter et al. found histologic evidence for reduction in perfusion of SCCVII squamous cell carcinoma tumors in nonanesthetized C3H/He mice [30]. Using LDF in anesthetized mice they found a

70% reduction in blood flow in the same tumor model following the administration of HYZ at 10 mg/kg. In a study by Zlotecki et al. the blood flow measured by LDF decreased by 60% after administration of the same dose of HYZ as used in our study [32]. They used LS174T human colon adenocarcinoma xenografts in anesthetized SCID mice. Kalmus et al. also used LDF to measure the effect of HYZ at different doses on blood flow in FSaII fibrosarcoma in nonanesthetized C3Hf/Sed mice [14]. Their results show the dynamics of blood flow decrease after injection of HYZ very similar to our results. At a dose of 1.0 mg/kg the maximum decrease of blood flow was about 40% reached about 10 minutes after injection. At the same dose they observed partial increase in blood flow 40 minutes after injection. They also observed that small doses of HYZ (0.25 mg/kg) resulted in increase of blood flow in tumors. Horsman et al. demonstrated a 80% decrease in blood perfusion measured by LDF 10 to 15 minutes after application of HYZ at the same dose as ours [11]. At HYZ dose of 0.1 mg/kg, however, an increase in tumor blood flow was observed. Their tumor model was C3H/Tif mouse mammary carcinoma growing in C3D2F1/Bom mice and their measurements were performed in non-anesthetized mice. Lin et al. evaluated the effect of HYZ at doses from 0.5 to 10.0 mg/kg on blood flow in RIF-1 tumors in anesthetized C3H mice [16]. They used both $^{86}$Rb rubidium extraction technique and LDF for this purpose. They found decreased blood flow at all doses used including the 0.1 mg/kg dose which was reported to increase blood flow in [11].

Our measurements of $pO_2$ by means of the novel time-resolved luminescence-based optical oximetry showed that $pO_2$ decreased in 10 to 20 minutes after injection of HYZ by approximately 80% from the pretreatment level (Fig. 7(a)). This decrease is well correlated to the decrease in blood flow measured by LDF. These results are also in accordance with the results of Okunieff et al. [18]. They showed that metabolic rate in FSaII fibrosarcoma tumors in C3Hf/Sed mice as measured by $^{31}$P-NMR spectroscopy was significantly decreased by HYZ at doses larger than 0.5 mg/kg but increased at a dose of 0.25 mg/kg. The decreased metabolism caused by the lack of oxygen was demonstrated by the dose-dependent decrease of organic phosphates and increase of inorganic phosphates.

Hydralazine is an effective peripheral vasodilator which has been used for treatment of hypertension in humans [1]. It is known to act by directly relaxing arteriolar smooth muscle and thus effectively reducing the peripheral vascular resistance. The result is a decreased blood pressure. Hydralazine on the other hand does not impair the cardiovascular reflexes. The decreased blood pressure also induces the compensatory effects of the increase in heart rate and stroke volume of the heart thus resulting in increased cardiac output and venous return. These effects, which counteract the effect of the decreased blood pressure, are probably stronger than the effect of dilated peripheral vasculature at low doses of HYZ which could explain the experimental evidence for increased blood flow in tumors at very low doses of HYZ reported by some authors [11,14]. At doses of HYZ larger than about 0.5 mg/kg the results of other authors and our own results clearly show that blood flow to tumors is significantly decreased due to HYZ. This decrease is a result of the "steal phenomenon" effect where the organism tries to maintain normal blood flow in vital organs by stealing the blood flow from less vital organs or tissues such as tumors [18]. In tumors, this "steal" effect may even lead to collapse of some tumor blood vessels, presumably of the vessels located in regions of elevated tumor IFP [30].

A consequence that one can expect from reduced blood flow in tumors is a subsequent decrease in oxygenation of tumors. This was directly demonstrated in our study where blood flow and $pO_2$ decreased with very similar dynamics. The relative decrease in $pO_2$ was greater and slightly faster than the relative decrease in blood flow. Experimental evidence was reported that application of hydralazine can enhance the antitumor effectiveness of different hypoxic cell-specific therapies such as hyperthermia and bioreductive drugs [6,11,14] which indirectly shows that oxygenation must have been reduced due to

hydralazine. HYZ has also been shown to reduce interstitial fluid pressure (IFP) in some solid tumors [21, 32]. Abnormally high tumor IFP in comparison to normal tissues has been recognized as one of the major obstacles for successful drug delivery in chemotherapy treatment of tumors [2,13,17]. Therefore it is believed that a blood flow-modifying agent which reduces IFP, such as hydralazine, might improve delivery of chemotherapeutic drug to tumor cells and thus enhance the antitumor effect of chemotherapy. The other recognized obstacle for successful drug delivery is poor blood perfusion in tumors which also contributes to development of hypoxia in tumors. Even though elevated tumor IFP was found to be associated with low oxygenation and resistance of tumors to radiotherapy [17], it is not clear if elevated IFP causes and contributes to development of tumor hypoxia through changes in blood flow. It has been speculated by some researchers that increased IFP in tumors may lead to vascular collapse of some capillaries and thus to development of hypoxia [17]. On the other hand some authors found no general correlation between IFP and oxygen partial pressure in solid tumors [3]. Therefore it might be that high IFP and poor blood perfusion and oxygenation are not directly correlated but simply associated in some tumors.

In our study we produced direct evidence of markedly decreased tumor oxygenation caused by decrease in blood perfusion after injection of hydralazine at a dose of 2.5 mg/kg. Even though hydralazine administered at the same dose also reduces IFP in solid tumors, it would probably not enhance drug delivery in tumors because of the counteracting effect of decreased tumor blood flow.

### *4.2. On validity of $pO_2$ measurements*

The time-resolved luminescence-based optical method, which we used in our study, is a new method which has only recently become available on the market. For this reason its use has been reported by few authors so far, e.g. [4,5,7,8,24]. Most of these works report on evaluation of the new method by comparing this method to other techniques, in particular to the well-established polarographic method. By many researchers the polarographic oximetry and its implementation in Eppendorf Histograph instrument is considered a "golden standard" for $pO_2$ measurements both in experimental and clinical tumors. A generally good correlation between the results of polarographic method and the new optical method was found in tumors [7,24] but there are also indications of discrepancies between the two methods in certain conditions and in different tissues [4,24], which arise from inherent differences between the two methods such as the underlying physical principle of measurement, dimension of the probe, tissue sampling volume, and consumption of oxygen by the sensor.

In our study, individual values of $pO_2$ measured at rest prior to injection of either HYZ or physiological saline showed great inter- and intratumoral variability in oxygenation. This is in good agreement with the long-known and well-documented inter- and intratumoral variability of many experimental and clinical tumors, e.g. [15,29]. Despite the relatively small number of individual measurements in our study ($n = 40$), the histogram of all measured $pO_2$ values at rest in Fig. 5 shows a commonly encountered distribution of $pO_2$ values in tumors with the majority of values close to zero $pO_2$. Also the response of $pO_2$ value measured during euthanasia of mice was as expected: $pO_2$ rapidly decreased to zero level. After application of HYZ, $pO_2$ decreased in all tumors with the dynamics similar to that of blood flow signal (Fig. 3). In a few cases we started the recording of $pO_2$ data before the probes were inserted into tumor. In all these cases $pO_2$ rapidly (within one minute) decreased from the initially high level (data not shown). This initial decrease is mostly a result of rapid consumption by the tissue of atmospheric oxygen brought into tissue by the insertion of the probe. This kind of response is also typically seen in well established polarographic oximetry. All these findings indicate that $pO_2$ measurements performed with the novel optical method were valid.

One feature found in our $pO_2$ measurements, however, was not expected. As described in the Results section, in all measurements where $pO_2$ did not remain at zero level after the initial decrease, the so-called type I $pO_2$ measurements, it took approximately 20 to 30 minutes for the readings to stabilize (see Figs 2 and 3). To our knowledge the unexpected multi-phase behavior of $pO_2$ has so far only been reported by Seddon et al. [24]. It is not clear whether other authors using OxyLite system have also observed this phenomenon or not.

Seddon et al. measured $pO_2$ in P22 rat carcinoma xenografts growing in the back of SCID mice [24]. In their study the mice were physically restrained but not anesthetized. OxyLite and Eppendorf Histograph instruments were used simultaneously in the same tumors. In case of OxyLite they observed the same two distinct types of $pO_2$ measurements as we did. In about 60% of their measurements they observed a rapid decrease of $pO_2$ to a nadir after insertion of the probe into tumor, followed by a slower increase to a higher and stable value within 20 minutes after the start of measurements (type I). They called this behavior a two-phase phenomenon. In about 40% of their measurements they only observed a rapid initial decrease to a nadir and no further increase afterwards (type II). They found both types of measurement even within the same tumor. They also observed that while the $pO_2$ in type I measurements responded to inhalation of carbon monoxide ($pO_2$ decreased), there was no response in type II measurements.

Observations by Seddon et al. are in perfect agreement with our own results: we found type I behavior in 19 out of 24 complete measurements and type II behavior in the remaining 5 measurements. We found both types within the same tumor as they did. In addition, in type I measurements, $pO_2$ decreased after injection of HYZ while there was no change after injection of HYZ in type II measurements in our study. This is comparable to the effect of carbon monoxide on $pO_2$ in their study. There is only one significant difference between our results and the results of Seddon et al. In our study $pO_2$ decreased initially after insertion of the probes to zero or close to zero value. In the study of Seddon et al. they call this an "early" $pO_2$ value as opposed to a "late" $pO_2$ value reached after the signal has stabilized. In their study the "early" $pO_2$ values were not necessarily zero or close to zero. At least two explanations are possible for this difference between our and their results. First, our mice were anesthetized while theirs were not. Second, the two tumor models in our and their studies may have intrinsically different physiological properties.

At the moment we do not have a definitive explanation for the described behavior of the $pO_2$ signals. One although unlikely but possible reason for the observed phenomenon could be related to the effect of the anesthetics used in our study. This explanation can now be ruled out since Seddon et al. observed the same characteristics of $pO_2$ signals in non-anesthetized mice [24]. Nevertheless, anesthesia undoubtedly does affect various parameters of physiological conditions in mice. Isoflurane used in our study is a recommended anesthetic for use in small animals due to its minimum side effects, stable anesthesia, and wide safety margin. Isoflurane produces little or no depressant effect on cardiovascular system but it causes some respiratory depression [27]. Nitrous oxide, which was used together with oxygen to deliver isoflurane to anesthetized mice, has no significant effects on cardiovascular or respiratory system [27]. Two mice included in our study were left anesthetized for 30 minutes before the $pO_2$ probes were inserted and the recording of data started. This was in contrast to the rest of mice where $pO_2$ probes were inserted and the recording started within four minutes after the start of anesthesia. No difference was seen. The time needed for $pO_2$ to stabilize in tumors of these two mice was in the same region of 20 to 30 minutes after insertion of the probes as in other mice.

The second and the most probable explanation for the observed phenomenon is the direct effect of the probe insertion on tissue oxygenation. Steinberg et al. evaluated the injury caused by insertion of polarographic $pO_2$ probe into tumor tissue [28]. The diameter of their probe (330 $\mu$m) was similar

to that of our probe (230 $\mu$m). Histological examination of tumor tissue around the insertion channel in tumor tissue revealed evidence of tissue destruction caused by the insertion of probe. Extravasated red blood cells were found at distances between 50 and more than 1000 $\mu$m from the insertion tracks depending on the tumor model. Tissue destruction was much less evident in normal muscle tissue in their study. Schramm et al. have provided histological evidence for compressed microvessels in vicinity of the tip of polarographic sensor inserted into rat muscle tissue [23]. The reduction of capillary lumen found within a 70 $\mu$m thick layer around the probe was as high as 50%. This reduction in lumen and flattening of capillaries results in increased perfusion resistance for the passing erythrocytes. The ultimate consequence is severely decreased oxygen-carrying capacity of capillaries and a decrease in oxygenation of tissue in direct contact with the probe.

Insertion of the probe undisputedly causes trauma to tissue in immediate vicinity of the insertion channel. Micro vessels can be torn and capillary occlusion can appear. All this can be expected to affect tissue $pO_2$ at the probe's tip. Based on our data and on evidence from other authors it is our hypothesis that when the probe is inserted, the tissue in its immediate vicinity is initially deoxygenated due to vessel compression and otherwise disrupted microcirculation. We speculate that during the time that follows, this tissue might be re-oxygenated either due to certain restoration of microcirculation or by diffusion of oxygen from the surrounding areas not affected by the insertion of the probe. Seddon et al. offer a similar explanation for their results [24]. They also speculate that the final stable $pO_2$ level reached after the initial transient decrease in type I $pO_2$ measurements (top graphs in Figs 2 and 3) is identical to the true tumor $pO_2$ value before insertion of the probes. This hypothesis however still needs further confirmation. In our study, for example, the median $pO_2$ value from 40 locations in 28 tumors was 10.3 mmHg which is twice as much as the median $pO_2$ measured by EPR oximetry and reported for the same tumor model as the one used in the present study [25].

The phenomenon described above was not seen in blood flow data measured by LDF but it was seen in all type I $pO_2$ measurements. This apparent difference between otherwise well correlated LDF and $pO_2$ measurements could be explained by a much larger tissue sampling volume in case of LDF measurement in comparison to $pO_2$ measurement. LDF samples a tissue volume of a cubic millimeter or more. The actual tissue sampling volume for the described time-resolved luminescence-based method is unknown but it is most probably much smaller than tissue sampling volume of LDF. The measured $pO_2$ values reflect local oxygenation in a very small part of tissue surrounding the probe tip. It is believed that this method samples the $pO_2$ in the cells and intercellular space in direct contact with the ruthenium probe tip, which means a volume of about 400 cells only [8]. The tissue surrounding the insertion channel, which is not affected by the insertion itself, should therefore contribute significantly to the LDF signal but not to the $pO_2$ signal.

## 5. Conclusion

In our first study using a novel time-resolved luminescence-based method for measurement of tissue oxygenation in combination with laser Doppler flowmetry we have shown that hydralazine at a dose 2.5 mg/kg significantly reduces both blood flow and oxygenation in experimental solid tumors. These results confirm that hydralazine might be used to potentiate the effectiveness of different hypoxic cell-specific therapies such as hyperthermia and bioreductive drugs. Our results are in good agreement with the results of other authors. Both optical methods were effective in showing that tumor hypoxia was rapidly increased by hydralazine. Both methods can be used successfully to monitor local changes in tumor blood flow and oxygenation which occur shortly after treatment. Interpretation of some aspects of the $pO_2$ data however requires further investigation.

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## References

[1] R.F. Albrecht, E.T. Toyooka, S.L.H. Polk and B. Zahed, Hydralazine therapy for hypertension during the anesthetic and postanesthetic periods, *International Anesthesiology Clinics* **16** (1978), 299–312.
[2] L.T. Baxter and R.K. Jain, Transport of fluid and macromolecules in tumors 1. Role of interstitial fluid pressure and convection, *Microvascular Research* **37** (1989), 77–104.
[3] Y. Boucher, I. Lee and R.K. Jain, Lack of general correlation between interstitial fluid pressure and oxygen partial pressure in solid tumors, *Microvascular Research* **50** (1995), 175–182.
[4] R.D. Braun, J.L. Lanzen, S.A. Snyder and M.W. Dewhirst, Comparison of tumor and normal tissue oxygen tension measurements using OxyLite or microelectrodes in rodents, *American Journal of Physiology. Heart and Circulatory Physiology* **280** (2001), H2533–H2544.
[5] J. Bussink, J.H.A.M. Kaanders, A.M. Strik and A.J. van der Kogel, Effects of nicotinamide and carbogen on oxygenation in human tumor xenografts measured with luminescence based fiber-optic probes, *Radiotherapy and Oncology* **57** (2000), 21–30.
[6] D.J. Chaplin and B. Acker, The effect of hydralazine on the tumor cytotoxicity of the hypoxic cell cytotoxin RSU-1069: evidence for therapeutic gain, *International Journal of Radiation Oncology, Biology, Physics* **13** (1987), 579–585.
[7] D.R. Collingridge, W.K. Young, B. Vojnovic, P. Wardman, E.M. Lynch, S.A. Hill and D.J. Chaplin, Measurement of tumor oxygenation: a comparison between polarographic needle electrodes and a time-resolved luminescence-based optical sensor, *Radiation Research* **147** (1997), 329–334.
[8] J.R. Griffiths and S.P. Robinson, The OxyLite: a fibre-optic oxygen sensor, *British Journal of Radiology* **72** (1999), 627–630.
[9] M.R. Horsman, Measurement of tumor oxygenation, *International Journal of Radiation Oncology, Biology, Physics* **42** (1998), 701–704.
[10] M.R. Horsman, M. Nordsmark and J. Overgaard, Techniques to assess the oxygenation of human tumors, *Strahlentherapie und Onkologie* **174**(IV) (1998), 2–5.
[11] M.R. Horsman, K.L. Christensen and J. Overgaard, Relationship between the hydralazine-induced changes in murine tumor blood supply and mouse blood pressure, *International Journal of Radiation Oncology, Biology, Physics* **22** (1992), 455–458.
[12] M. Höckel, B. Vorndran, K. Schlenger, E. Baußmann and P.G. Knapstein, Tumor oxygenation: a new predictive parameter in locally advanced cancer of the uterine cervix, *Gynecologic Oncology* **51** (1993), 141–149.
[13] R.K. Jain, Determinants of tumor blood flow: a review, *Cancer Research* **48** (1988), 2641–2658.
[14] J. Kalmus, P. Okunieff and P. Vaupel, Dose-dependent effects of hydralazine on microcirculatory fuction and hyperthermic response of murine FSaII tumors, *Cancer Research* **50** (1990), 15–19.
[15] M.-C. Kavanagh, A. Sun, Q. Hu and R.P. Hill, Comparing techniques of measuring tumor hypoxia in different murine tumors: Eppendorf $pO_2$ Histograph, [3H]Misonidazole binding and peired survival assay, *Radiation Research* **145** (1996), 491–500.
[16] J.-C. Lin and C.W. Song, Effects of hydralazine on the blood flow in RIF-1 tumors and normal tissues in mice, *Radiation Research* **124** (1990), 171–177.
[17] M.F. Milosevic, A.W. Fyles and R.P. Hill, The relationship between elevated interstitial fluid pressure and blood flow in tumors: a bioengineering analysis, *International Journal of Radiation Oncology, Biology, Physics* **43** (1999), 1111–1123.
[18] P. Okunieff, C.S. Walsh, P. Vaupel, F. Kallinowski, B.M. Hitzig, L.J. Neuringer and H.D. Suit, Effects of hydralazine on *in vivo* tumor energy metabolism, hematopoietic radiation sensitivity, and cardiovascular parameters, *International Journal of Radiation Oncology, Biology, Physics* **16** (1989), 1145–1148.
[19] Oxford Optronix, *OxyLite user manual ver. 1.12.*, Oxford Optronix Ltd., Oxford, 1998.
[20] Oxford Optronix, *OxyFlo user manual ver. 1.03.*, Oxford Optronix Ltd., Oxford, 2000.
[21] B. Podobnik and D. Miklavčič, Influence of hydralazine on interstitial fluid pressure in experimental tumors – a preliminary study, *Radiology and Oncology* **34** (2000), 59–65.

[22] J.A. Raleigh, M.W. Dewhirst and D.E. Thrall, Measuring tumor hypoxia, *Seminars in Radiation Oncology* **6** (1996), 37–45.
[23] U. Schramm, W. Fleckenstein and C. Weber, Morphological assessment of skeletal muscular injury caused by $pO_2$ measurements with hypodermic needle probes, in: *Clinical oxygen measurement II,* A.M. Ehrly, W. Fleckenstein, J. Hauss and R. Huch, eds, Blackwell Ueberreuter Wissenschaft, Berlin, 1990, pp. 38–50.
[24] B.M. Seddon, D.J. Honess, B. Vojnovic, G.M. Tozer and P. Workman, Measurement of tumor oxygenation: *in vivo* comparison of a luminescence fiber-optic sensor and a polarographic sensor and a polarographic electrode in the P22 tumor, *Radiation Research* **155** (2001), 837–846.
[25] G. Sersa, M. Krzic, M. Sentjurc, T. Ivanusa, K. Beravs, M. Cemazar, M. Auersperg and H.M. Swartz, Reduced tumor oxygenation by treatment with vinblastine, *Cancer Research* **61** (2001), 4266–4271.
[26] Laser-Doppler blood flowmetry, A.P. Shepherd and P.A. Oberg, eds, Kluwer Academic Publishers, Boston, 1989.
[27] C.E. Short, R.R. Paddleford and R.C. Harvey, Induction and maintenance of general anaesthesia in small animal practice, *Wiener tierärztliche Monatsschrift* **80** (1993), 177–187.
[28] F. Steinberg, E. Hildenhagen-Brüggemann and M.A. Konerding, Oxygen electrode injury in tumour tissue, in: *Tumor oxygenation,* O. Thews, D.K. Kelleher and P.W. Vaupel, eds, Gustav Fischer Verlag, Stuttgart, 1995, pp. 186–193.
[29] O. Thews, D.K. Kelleher and P.W. Vaupel, Intra- and inter-tumor variability of oxygenation: quantification and evaluation of minimum number of electrode tracks for representative measurements, in: *Tumor oxygenation,* O. Thews, D.K. Kelleher and P.W. Vaupel, eds, Gustav Fischer Verlag, Stuttgart, 1995, pp. 39–47.
[30] M.J. Trotter, B.D. Acker and D.J. Chaplin, Histological evidence for nonperfused vasculature in a murine tumor following hydralazine administration, *International Journal of Radiation Oncology, Biology, Physics* **17** (1989), 785–789.
[31] P. Vaupel, D.K. Kelleher and O. Thews, Modulation of tumor oxygenation, *International Journal of Radiation Oncology, Biology, Physics* **42** (1998), 843–848.
[32] R.A. Zlotecki, L.T. Baxter, Y. Boucher and R.K. Jain, Pharmacologic modification of tumor blood flow and interstitial fluid pressure in a human tumor xenograft: network analysis and mechanistic interpretation, *Microvascular Research* **50** (1995), 429–443.