Reduced Tumor Oxygenation by Treatment with Vinblastine

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ABSTRACT

Vinblastine (VLB) previously has been shown to perturb tumor blood flow, but the effect of these perturbations on tissue oxygenation is not known. The recent development of electron paramagnetic resonance (EPR) oximetry now has made it feasible to measure the effects of changes of perfusion on the pO2, in tumors and normal tissues as a function of time and dose. We measured changes in tumor perfusion by Patent blue staining, tumor blood volume and microvascular permeability by contrast-enhanced magnetic resonance imaging, and tumor oxygenation by EPR in s.c. SA-1 murine tumors. We found that treatment with VLB induced dose-dependent reduction in tumor perfusion. One hour after i.p. treatment of mice with 2.5 mg/kg VLB, tumor perfusion was reduced to 20% of the pretreatment value and returned to close to original values within 48 h. A transient tumor blood flow-modifying effect of VLB was demonstrated also by contrast-enhanced magnetic resonance imaging; reduction of tumor blood volume and microvascular permeability was found. Reduced tumor oxygenation was found as measured by EPR oximetry, with the same time course of changes in tumor blood flow. Tumor oxygenation was reduced to 50% of pretreatment value 1 h after the treatment with 2.5 mg/kg VLB and returned to pretreatment levels within 24 h after the treatment. Although the directions of the changes in perfusion and oxygenation were similar, they were quantitatively different. Reduction in oxygenation of normal tissues, muscle, and subcutis also occurred but was smaller and returned to pretreatment values more quickly compared to the changes induced in the tumors. In conclusion, the present study demonstrates that VLB causes a profound reduction in tumor blood flow and oxygenation, which may have implications in controlling side effects of therapy and the planning of combined treatment with VLB, either with other chemotherapeutic drugs or with radiotherapy.

INTRODUCTION

Knowledge about tumor physiology is important for understanding of tumor growth as well for rational planning of tumor treatment (1). The vascular supply and tumor oxygenation are especially important for tumor growth (2). Reduction in tumor blood flow can lead to an increase in hypoxia and extracellular acidification (2, 3). Additionally, if blood flow is chronically impaired, a cascade of tumor cell death will occur because of the lack of nutrients and accumulation of catabolite products (4). Therefore, tumor vasculature has become a potential target for cancer treatment. Two approaches have become feasible, antiangiogenic therapy hindering the neovascularization of the tumor tissue (5) and vascular targeted therapy affecting the existing vascularization of the tumors (6).

Many anticancer agents and therapies in current use have been shown to exert their antitumor action, to some extent, as a direct consequence of compromising vascular function (2). Even if these therapies induce only transient reduction in tumor blood flow, this can be exploited in combination with bioreductive agents or in optimal combination with other therapies (7). These agents include hyperthermia, photodynamic therapy, high-energy shock waves, cytokines, such as tumor necrosis factor-α and interleukin 1α, drugs such as hydralazine, serotonin, flavone acetic acid, vincristine, alkaloids, combretastatin, and application of high-voltage electric pulses (2, 8). However, the potential of modifying tumor blood flow in the clinic with many of the agents is limited by several factors, including unacceptable toxicity. Nevertheless, studies in experimental systems with such agents have demonstrated that blood flow effects can be exploited to improve therapeutic outcome. Application of these agents in combination with bioreductive drugs or other treatment approaches that are effective in lower oxygen pressure has already been demonstrated (9, 10). There is thus a need to obtain quantitative data on the effects of drugs on the vascular system and oxygenation under in vivo conditions, so that their use can be translated into the clinical setting.

VLB, a Vincristine, is a chemotherapeutic drug that is used in combined treatment of testis tumors, Hodgkin’s and non-Hodgkin’s lymphomas, breast carcinomas, gastric carcinomas, squamous cell carcinomas, and many others (11–13). The cytotoxic action of VLB is predominantly by interference with the polymerization of tubulin and induction of mitotic cell death (14). A cytotoxic effect of VLB in interphase was described with VLB doses higher than those that induce mitotic arrest (15). It has been shown that VLB compromises tumor vasculature to some extent, which contributes to its antitumor effectiveness (16, 17). In addition to its cytotoxic effects, VLB increases cell membrane fluidity, which can be exploited for increased drug delivery into the cells (18, 19).

The effect of VLB on tumor blood flow, a transient reduction in tumor blood supply, has already been described previously (16). However, the antivascular action of VLB is not fully understood. With the new techniques that now are available, it is possible to measure temporal and spatial changes in tumor and normal tissue oxygenation by EPR oximetry (20–23), as well as changes in tumor BV and microvascular permeability by dynamic contrast-enhanced MRI (24–28). The aim of this study was to measure the time course of tumor blood flow changes induced by treatment with VLB and its effect on tumor oxygenation.

MATERIALS AND METHODS

Mice and Tumors. The animals used in the present experiments were male and female A/J mice purchased from Rudjer Boškovic Institute (Zagreb, Croatia). They were maintained at 22°C with a natural day/night light cycle in a conventional animal colony and fed food and water ad libitum. The mice were 10–14 weeks old at the beginning of the experiments. The tumor used was fibrosarcoma SA-1 (The Jackson Laboratory, Bar Harbor, ME). SA-1 cells were obtained from the ascitic form of the tumors in mice, which were serially transplanted once per week. Subcutaneous tumors were implanted by injecting 105 viable tumor cells under the skin on the rear dorsum. Six to 8 days after implantation, the tumors reached 45 mm3.
in volume (6 mm in diameter) and mice were randomly divided into experimental groups consisting of at least six mice. For MRI and EPR oximetry, animals were anesthetized with a mixture of Domitor (1.0 mg/kg body weight; Pfizer GmbH, Karlsruhe, Germany) and 10% ketamine (75.0 mg/kg body weight; Veyx-Pharma GmbH, Schwarzenborn, Germany) administered i.p. During anesthesia, body temperature of the mice was kept at physiological values by a regulated heating pad on which the mice were placed (Guymar T/pump; Linton Instruments, Norfolk, United Kingdom).

Assessment of Antitumor Effect. Tumor growth was followed daily by measuring tumor diameter in three orthogonal directions (e_x, e_y, and e_z) using Vernier calipers, following treatment with VLB. Tumor volumes were calculated by the formula \( V = \frac{4}{3} \pi \times e_x \times e_y \times e_z / 6 \). From the measurements, the arithmetic mean and SE were calculated for each experimental group. Tumor growth was reduced by the mean tumor doubling time of experimental groups compared to untreated tumors. VLB (Lilly France S.A., Fegersheim, France) was dissolved in 0.9% NaCl at different concentrations. Animals were treated with VLB i.p.

Assessment of Tumor Perfusion by Patent Blue Staining. Patent blue (Byk Gulden, Switzerland) was used to estimate tumor perfusion. Patent blue (1.25%) diluted in 0.2 ml physiological saline was injected at different time points into the tail vein of animals after VLB injection. After the dye was distributed evenly through the tissue (1 min), animals were sacrificed and tumors were carefully excised. The excised tumors were carefully removed from the skin, cut in half along their largest diameter, and immediately evaluated. The percentage of stained area of tumor cross-section (perfused) as opposed to nonstained area (nonperfused) was visually estimated by two individuals. The mean of both estimations was used as an indicator of tumor perfusion. Because of marked differences in tumor staining between well-perfused and poorly perfused tumors, visual estimation of the whole tumor cross-sections gives good estimation and reliable results. In the study comparing Patent blue staining and the pharmacological method measuring relative tumor blood flow, good correlation between both methods was found (r = 0.962) (29). Briefly, \(^{18}\)RBC extraction technique gives measure of tissue perfusion as a function of cardiac output and is expressed as percent injected activity per gram or relative uptake (percent control) (29).

Dynamic Contrast-enhanced MRI. This technique was used to assess changes in tumor BV and microvascular PS area product within the first 1.5 h after treating animals with VLB that was administrated in a dose of 2.5 mg/kg 10 min prior to contrast-enhanced MRI. To perform contrast-enhanced MRI gadomer-17 (Shering AG, Berlin, Germany) was used as a representative of macromolecular magnetic resonance contrast agents. When administered i.v., this agent does not diffuse through endothelia, unless there is a pathological change (27, 30). The size of the agent is approximately 30 kDa, which classifies it as intermediate-sized agent. One of the advantages of such size is complete renal elimination. The agent was administered in a dose of 0.025 mmol/kg in a bolus via a 23-gauge i.v. cannula (Vygon 247 Venoflux infusion set; France) that was inserted into a tail vein of anesthetized mice. No systemic toxicity was observed, and the procedure was well tolerated by the animals.

Contrast-enhanced MRI was performed on a 2.35 T Bruker Biospec system with a horizontal bore magnet. A precontrast image (complete k-space data set) was acquired first, using a standard spin-echo technique. Imaging parameters were: repetition time, \( T_R = 600 \text{ ms} \); echo time, \( T_E = 18 \text{ ms} \); matrix, \( 256 \times 256 \); slice thickness, 2 mm; field of view, 7 cm; and acquisition time, 5 min. Second, gadomer-17 was administered, and a central data subset of the k-space domain (in the phase-encoding direction) with dimensions 32 \( \times 256 \) k-space data points was acquired repetitively for 60–100 min (80–100 “key” images). Each key image was acquired with 32 phase-encoding steps that took 38 s. Before the reconstruction, the subset was first completed in remaining k-space points (which were not included in temporal acquisition) with the data from the precontrast acquisition. Finally, images were reconstructed with two-dimensional inverse Fourier transformation.

To obtain a measure for altered blood flow (PS and BV), the magnetic resonance signal was measured within the tumor and vena cava in precontrast image and at least 80 postcontrast images that were acquired every 38 s. The signal was corrected for signal variations against a water phantom. From the measured signal, the concentration, \( C_p \), of the gadomer-17 in the tumor was calculated by subtraction of the precontrast signal from the postcontrast signals on a pixel-by-pixel basis. The concentration of gadomer-17 in a slow-flowing vessel, such as the inferior vena cava, \( C_{p,c} \), was obtained in a similar way. The linearity of the \( C_p / C_{p,c} \) fit was checked for the first 30–50 points. PS and BV were calculated using an established method (24–27, 30). PS was calculated as:

\[
PS = PS'(1 - Hct)
\]

where \( Hct \) is the measured hematocrit of the blood (47% for tumors in animals) and \( PS' \) linear fit coefficient of \( C_p / C_{p,c} \) at different time points. BV was calculated as:

\[
BV = \frac{C_p(t)}{C_{p,c}(t)} - PS't
\]

where \( C_p \) is concentration of contrast agent in tumor and \( C_{p,c} \) is concentration of contrast agent in blood.

EPR Oximetry. EPR oximetry was used to evaluate tumor oxygenation as well as changes in \( pO_2 \) in normal muscle tissue and subcutis after treatment of animals with VLB. EPR oximetry is noninvasive method, which allows monitoring of \( pO_2 \) repeatedly at the same point in the tissue over long periods of time. For this purpose, a paramagnetic probe, which is sensitive to oxygen, is implanted into the desired place in the tissue and its EPR spectral line width measured with time after the injection of VLB. The method is based on the fact that molecular oxygen is paramagnetic and causes fast relaxation of the other paramagnetic centers in the immediate surrounding (31). Therefore, in the presence of oxygen, the line width of the EPR lines is broadened; the extent of broadening depends on \( pO_2 \) (20, 22). The \( pO_2 \) in the region in contact with the probe is measured with this technique.

A particulate paramagnetic material, a char of the Bubinga tree (Bubinga), was used to assess the partial oxygen pressure in tumors and selected normal tissues. In the same animal, the particles of the char were placed in two different locations: one in the selected site in the tumor (center or periphery) and the other in the selected normal tissue (skeletal muscle or subcutis). At the specific tissue, a small quantity of the char (=0.5 mm\(^3\)) with particle size 10 \( \mu \text{m} \) was injected when the tumors reached 6 mm in diameter (45 mm\(^3\)). One day after the insertion of the char, the line widths of the EPR spectra were measured with time after i.p. injection of VLB (2.5 mg/kg).

The EPR spectra were recorded continuously for at least 90 min. The measurements were performed on a Varian E-9 EPR spectrometer, with a custom-made low-frequency microwave bridge operating at 1.1 GHz and an extended loop resonator (11 mm in diameter), both designed by Prof. T. Walczak (Dartmouth Medical School, Hanover, NH). Typical spectrometer settings were: modulation frequency, 100 KHz; modulation amplitude not more than one-third of the peak-to-peak line width; and scan range, 2 mT. The line width of the EPR spectra reflects the partial pressure of oxygen (\( pO_2 \)), which was determined from an existing calibration curve (32).

Statistical Analysis. All data were tested for normality of distribution. The differences between the mean values of groups were tested for significance by a modified t test (Bonferroni t test) after one-way ANOVA was performed and fulfilled. A Pearson correlation coefficient was calculated to determine correlation between Patent blue staining and EPR oximetry. SigmaStat statistical software (SPSS Inc.) was used for statistical analysis.

RESULTS

Antitumor Effectiveness. The antitumor effectiveness of single i.p. treatments was tested on s.c. SA-1 tumors in mice at different VLB doses (Table 1). A gradual increase in antitumor effectiveness was observed with increasing doses of VLB (Table 1). Antitumor Effectiveness of VLB given i.p. on SA-1 tumors in A/J mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>DT* (days)</th>
<th>GD (days)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>1.4 ± 0.2</td>
<td>0.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>VLB, 1.25 mg/kg</td>
<td>6</td>
<td>1.5 ± 0.2</td>
<td>0.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>VLB, 1.75 mg/kg</td>
<td>6</td>
<td>1.6 ± 0.2</td>
<td>0.3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>VLB, 2.5 mg/kg</td>
<td>6</td>
<td>2.4 ± 0.4</td>
<td>0.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>VLB, 5.0 mg/kg</td>
<td>6</td>
<td>2.7 ± 0.2</td>
<td>1.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>VLB, 7.5 mg/kg</td>
<td>6</td>
<td>3.8 ± 0.3</td>
<td>2.4</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*DT, tumor doubling time (arithmetic mean ± SE); GD, tumor growth delay. *P vs. control.
was observed. Only the highest three doses induced significant tumor growth delay, but not >2.4 days (Table 1; Fig. 1). None of the VLB doses tested had side effects and all were well tolerated by the animals.

**Effects on Tumor Perfusion.** The VLB doses used in evaluation of antitumor effectiveness were also used for evaluation of tumor perfusion, using Patent blue tumor staining. Tumors without treatment were well perfused, with 84.2% of the tumor area being stained. Treatment of animals with VLB doses up to 1.25 mg/kg induced no significant reduction of the area of the tumor that was stained, whereas doses of 1.75 mg/kg and higher significantly reduced the stained area (Fig. 2). The data acquired 2 h after treatment with VLB clearly demonstrated that VLB doses higher than 1.25 mg/kg substantially reduce tumor perfusion. With the dose 2.5 mg/kg, there was 74% reduction in tumor perfusion at 2 h. Doses of 3.75 mg/kg and higher almost completely stopped tumor perfusion.

The time course of tumor perfusion changes was studied with two VLB doses, 1.75 and 2.5 mg/kg (Fig. 3). The onset of reduced tumor perfusion after VLB treatment was fast, maximal reduction was observed by 1 h after the treatment; thereafter, tumors gradually started to reperfuse. As already demonstrated, the reduction of tumor perfusion was dose dependent. In addition, the recovery of tumor perfusion was complete at 24 h after treatment with the dose of 1.75 mg/kg, whereas with the dose of 2.5 mg/kg, tumor perfusion had not completely recovered even at 48 h after treatment.

**Dynamic Contrast-enhanced MRI.** Dynamic contrast-enhanced MRI was used to evaluate changes in tumor BV and microvascular permeability within 1.5 h after the treatment with 2.5 mg/kg VLB. The contrast agent gadomer-17 enhanced the untreated tumors heterogeneously. The periphery of the tumor was enhanced more than the center of the tumor in the first minute after the injection of the contrast medium, indicating that the periphery was better vascularized than the center of the tumor. The tumor enhancement then increased gradually, indicating diffusion of the contrast medium from the blood into the interstitial space (Fig. 4A). Tumors treated with VLB showed delayed enhancement with gadomer-17 (Fig. 4B), indicating a change in blood flow or decreased permeability. The concentration of contrast in the tumor (C_T) was maximal ~40 min after the administration of the contrast agent (60 min after treatment; Fig. 5). Thereafter, the enhancement slowly decreased but remained above the baseline value (precontrast enhancement) for the entire measurement time (110 min).

**Tissue Oxygenation.** EPR oximetry was used to measure pO_2 in SA-1 tumors, leg muscle, and subcutis. The pO_2 values in normal tissues and untreated tumors were quite different. The pO_2 in muscle tissue was higher than in subcutis and tumor. In the tumor, the pO_2 was higher in the periphery (7.5 mm Hg) than in the center (5.4 mm Hg; Table 2).

Treatment of animals with VLB (2.5 mg/kg) significantly reduced pO_2 in all of the tissues. The greatest reduction was in the tumor (~52%). The decrease in pO_2 was less in muscle (~40%) and in subcutis pO_2 (~10%). Within 1 h after treatment with VLB, the tumors became hypoxic, whereas the muscle and subcutis still had pO_2 in the range that was probably sufficient for normal physiology (Table 2).

The time course of pO_2 changes in tumors, muscle, and subcutis was similar to the changes of perfusion in the tumor. VLB treatment very quickly reduced oxygenation of the tissues, with steady recovery of oxygenation after 1 h. The recovery was faster in muscle (after 8 h) than in the tumors (after 24 h; Fig. 6).
Correlation among the Tumor Perfusion, Contrast Agent Accumulation, and Oxygenation Changes. It was possible to evaluate the correlation among the perfusion, contrast agent accumulation, and oxygenation in the SA-1 tumors because they were measured at the same time points, on the same tumor model, and in the same tumor volume within 1–2 h after the i.p. treatment of animals with VLB (2.5 mg/kg). As already shown, the reduction in tumor perfusion, as measured by Patent blue staining, correlated well with the reduction in tumor BV and a reduction in microvascular permeability as measured by dynamic contrast-enhanced MRI. All three parameters reflect the blood supply of the tumors. As demonstrated in this study, the pattern of tumor oxygenation was similar to that of the blood supply of the tumors. A very high correlation was found between perfusion and pO$_2$ ($r = 0.724$). Maximal reduction in tumor perfusion and the lowest partial oxygen pressure was found 1 h after VLB treatment, with steady recovery thereafter. The pO$_2$ in the tumors recovered to the pretreatment value within 24 h, but as assessed by the Patent blue staining, the tumors did not fully reperfuse even after 48 h (Fig. 7).

DISCUSSION

This study shows that treatment with VLB reduces tumor oxygenation, with the same time course as reduced tumor blood flow. Dose-dependent reduction in tumor perfusion was found in the tumors within 1 h after VLB treatment, with steady recovery of perfusion over 48 h. Changes observed in tumor perfusion, as measured by Patent blue staining, were reflected in reduced tumor BV and decreased microvascular permeability of the tumors as measured by contrast-enhanced MRI. These parameters, which reflect tumor blood flow, correlated with the overall time course of pO$_2$ changes in the tumors measured by EPR oximetry.

Evaluation of tumor perfusion by the Patent blue staining method has already been shown to correlate with the $^{86}$Rb extraction technique (29). The method is simple and provides good estimation of tumor perfusion, especially in tumors with light colors, such as the SA-1 tumor in A/J mice that was used in this study. Treatment of animals with VLB was found to induce significant reduction in tumor perfusion within 1–2 h, but the tumors started to reperfuse very quickly. The maximum effects in animals required doses (2.5 mg/kg and higher) that are higher than adequate doses for humans (7.7...
In the tumors, microvascular abnormalities are usually associated with vascular leakage. Changes in endothelial cells of microvessels manifest as opening of endothelial tight junctions and increased vesicular transport in abnormally growing tumor endothelium (34). Formed intercellular gaps allow intravascular fluids and macromolecular solutes to leak into the interstitial space (35), which we demonstrated in vivo using dynamic contrast-enhanced MRI. The results of the present study are consistent with the results of previous studies, demonstrating that both VLB and vincristine cause a dramatic and prolonged decrease in blood flow in tumors, significantly greater than any change in normal tissue perfusion (16, 17, 33). On the basis of these observations and perfusion measurements on other tumors, it was demonstrated that Vinca alkaloids, in addition to their antitumor action by binding to intracellular protein tubulin, exert their antitumor action in part via impairment of blood flow (38). In that study, however, the doses that were shown to induce hemorrhagic necrosis of tumors were close to maximal tolerated doses, doses that are higher than used clinically, and doses that cause significant toxicity (16). This study shows that doses lower than maximal tolerated doses also have an antivascular effect. However, it was also shown that VLB doses lower than 1.25 mg/kg had no effect on tumor blood flow. On the basis of the data on tumor blood flow, it would be expected that with doses lower than 1.25 mg/kg there were no oxygen concentration changes in the tumors and probably even less in normal tissues.

A few studies have demonstrated that conventional chemotherapeutic drugs also can have a vascular component of damage and indicated that this is associated with vascular complications in the patients (39–42). Other chemotherapeutic drugs (cisplatin and bleomycin) whose mechanisms include vascular effects have toxicities that are similar to some of those seen with VLB (40). It is of importance that VLB induces reduced oxygenation not only in tumors but also in normal tissues; therefore, vascular episodes that have been described after or during VLB treatment may be ascribed to reduced heart oxygenation. In a series of patients with locally advanced (T4) thyroid carcinomas treated with infusion of very low doses of VLB (2 mg over 12 or 24 h), five times lower than standard bolus dose, transient angina-like chest pain was observed in some patients. No changes in electrocardiogram or “cardiac enzymes” could be detected in these patients. Furthermore, these complications are not to be expected only in patients with already known vascular disease, but also in patients without vascular history. In connection to this, it has to be considered that with decreased drug dosage, which will still affect tumor blood flow and oxygenation, less toxicity could be expected to normal tissue, since VLB exerts a blood-modifying effect selectively toward tumors compared to normal tissues.

EPR oximetry previously has been used in measurements of oxygenation of various tissues and tumors, as well as after other therapeutic approaches (37). It is expected that EPR oximetry will be clinically useful for optimizing cancer therapy by enabling it to be modified on the basis of the pO2 measured in the tumor and for monitoring the status and response to treatment of peripheral vascular disease (21, 22).

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Contrast-enhanced MRI, a noninvasive method, was used to confirm the results obtained by the Patent blue technique and to determine the temporal changes in tumor blood flow following treatment with VLB. Gadomer-17 is a macromolecular contrast agent that provides assessment of changes in tumor BV and microvascular permeability. Our results are not consistent with data showing increased vascular permeability in endothelial cells treated with VLB in in vitro conditions. Passage of FITC-dextran through endothelial cell monolayers was used as a marker of permeability changes. VLB as well as combretastatin, another tubulin-binding agent with an antivascular mechanism of action, increased permeability of endothelial cells (36). In addition, it was shown that treatment with VLB caused a rearrangement of actin filaments, which leads to cell shape change, a formation of gaps between the cells, and consequently to increased permeability (36), which we demonstrated in vivo using dynamic contrast-enhanced MRI. These different results may reflect real differences between the systems that we studied or may reflect the effect of decreased perfusion, which also would result in less localization of the contrast material because of decreased delivery.

By the use of EPR oximetry, we were able to directly demonstrate the correlation between the reduction in partial oxygen pressure in the tumors and the tumor blood flow-modifying effect of VLB. To our knowledge, this is the first study demonstrating reduced oxygenation in the tumors induced by VLB. We found that reduction of pO2 in tumors was greater compared to that in selected normal tissues. EPR oximetry previously has been used in measurements of oxygenation of various tissues and tumors, as well as after other therapeutic approaches (37). It is expected that EPR oximetry will be clinically useful for optimizing cancer therapy by enabling it to be modified on the basis of the pO2 measured in the tumor and for monitoring the status and response to treatment of peripheral vascular disease (21, 22).

4 M. Auersperg, personal communication.
providing information for optimal planning of the combined treatment.

It should be noted that although the effects of VLB on perfusion and oxygenation have the same general time course, neither the quantitative details of the time course of the changes nor the absolute magnitude of the changes are the same. Therefore, direct measurement of the pO₂ may be needed to obtain data that are needed for determining the times of maximum vulnerability for damage and for optimal combined therapy.

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