Validation of fluorescent-labeled microspheres for measurement of relative blood flow in severely injured lungs

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1Department of Anesthesiology and Intensive Care, Carl Gustav Carus University Hospital, 01307 Dresden, Germany; Departments of 2Anesthesiology, 3Physiology and Biophysics, and 4Medicine, University of Washington School of Medicine, Seattle, Washington 98195; and 5The Mountain-Whisper-Light Statistical Consulting, Seattle, Washington 98112

Hübner, Matthias, Jennifer E. Souders, Erin D. Shade, Michael P. Hlastala, Nayak L. Polissar, and Robb W. Glenny. Validation of fluorescent-labeled microspheres for measurement of relative blood flow in severely injured lungs. J. Appl. Physiol. 87(6): 2381–2385, 1999.—The aim of the study was to validate a nonradioactive method for relative blood flow measurements in severely injured lungs that avoids labor-intensive tissue processing. The use of fluorescent-labeled microspheres was compared with the standard radiolabeled-microsphere method. In seven sheep, lung injury was established by using oleic acid. Five pairs of radio- and fluorescent-labeled microspheres were injected before and after established lung injury. Across all animals, 175 pieces were selected randomly. The radioactivity of each piece was determined by using a scintillation counter. The fluorescent dye was extracted from each piece with a solvent without digestion or filtering. The fluorescence was determined with an automated fluorescent spectrophotometer. Perfusion was calculated for each piece from both the radioactivity and fluorescence and volume normalized. Correlations between flow determined by the two methods were in the range from 0.987 ± 0.007 (SD) to 0.991 ± 0.002 (SD) after 9 days of soaking. Thus the fluorescent microsphere technique is a valuable tool for investigating regional perfusion in severely injured lungs and can replace radioactivity.

METHODS

The study was approved by the University of Washington Animal Care Committee.

Animal preparation. The experiments were performed on seven adult sheep of both genders, ranging in weight from 22.6 to 27.3 kg. The animals were premedicated with 0.6 mg/kg xylazine (Xyla-Ject, Phoenix Pharmaceutical, St. Joseph, MO), anesthetized with thiopental sodium (20 mg/kg), intubated, and ventilated with a servo ventilator 900 C (Siemens, Solna, Sweden). Anesthesia was maintained by using a continuous infusion of thiopental sodium titrated to suppress hemodynamic responses to noxious stimuli. Once anesthetic depth was adequate, the animals were paralyzed using a continuous infusion of thiopental sodium (11). Because severely injured lung tissue is more like solid organs than normal lung tissue, digestion and filtration of lung pieces may be necessary.

The primary purpose of this study was to validate the use of fluorescent-labeled microspheres for estimating organ perfusion in severely injured lungs. Fluorescent dye was extracted from dried lung pieces, thus avoiding the time-consuming tissue digestion and filtration procedures.

The use of fluorescent-labeled microspheres for measurement of regional lung perfusion is a well-established method (7). When the number of injected microspheres is sufficiently large, and when appropriately sized microspheres are used, regional blood flow is proportional to the number of trapped microspheres (2). Our laboratory (7) showed that the extraction of the fluorescent dye can be simplified with the use of an automated spectrophotometer. We further simplified the tissue processing by avoiding digestion and filtration of the lung pieces, while still yielding excellent correlations. However, retrieving fluorescent microspheres from solid organs requires many labor-intensive steps. Radiolabeled microspheres are easier to use but pose health risks, require special precautions for use and disposal, have limited shelf lives, and are relatively expensive. There are few reports about the use of nonradiolabeled microspheres in injured lungs (11). Because severely injured lung tissue is more like solid organs than normal lung tissue, digestion and filtration of lung pieces may be necessary.

The experiments were performed on seven adult sheep of both genders, ranging in weight from 22.6 to 27.3 kg. The animals were premedicated with 0.6 mg/kg xylazine (Xyla-Ject, Phoenix Pharmaceutical, St. Joseph, MO), anesthetized with thiopental sodium (20 mg/kg), intubated, and ventilated with a servo ventilator 900 C (Siemens, Solna, Sweden). Anesthesia was maintained by using a continuous infusion of thiopental sodium titrated to suppress hemodynamic responses to noxious stimuli. Once anesthetic depth was adequate, the animals were paralyzed using a continuous infusion of thiopental sodium (11). Because severely injured lung tissue is more like solid organs than normal lung tissue, digestion and filtration of lung pieces may be necessary.
prone. After an initial stabilization period during which the respiratory minute volume was adjusted to maintain arterial P\textsubscript{CO}\textsubscript{2} between 36 and 46 Torr, baseline measurements were recorded. Lung injury was induced by emulsifying 15 ml of previously extracted blood with 0.14–0.23 ml OA/kg (C\textsubscript{18}H\textsubscript{34}O\textsubscript{2}, Sigma Chemical, St. Louis, MO) and injecting it with continuous shaking over 30–45 min. Severe lung injury was considered established when the clinical criteria of the definition of the American/European consensus conference on acute respiratory distress syndrome (ARDS) were fulfilled (ratio of arterial P\textsubscript{O}\textsubscript{2} to inspiratory O\textsubscript{2} fraction < 200, pulmonary capillary wedge pressure < 19 Torr) (3). The animals were then studied for 120 min after the injury was established.

Microsphere validation. Details of the methods are described by Glenny et al. (7). Briefly, five fluorescent polystyrene microspheres (red, orange, blue-green, yellow-green, and crimson) of 15 µm diameter (FluoSpheres, Molecular Probes, Eugene, OR) and five radiolabeled styrene-divinyl benzene microspheres ([\textsuperscript{144}Ce, \textsuperscript{113}Sn, \textsuperscript{103}Ru, \textsuperscript{95}Nb, and \textsuperscript{46}Sc]) of 15.5 µm diameter (DuPont NEN Research Products, Boston, MA) were used. The time points of injection were baseline (t\textsubscript{base}), established injury (t\textsubscript{0}), 30 min (t\textsubscript{30}), 60 min (t\textsubscript{60}), and 120 min (t\textsubscript{120}) after established injury. Immediately before injection, the microspheres were vortexed, sonicated for 90 s, and combined in a single syringe. Fluorescent (1.5 × 10\textsuperscript{6}) and radiolabeled microspheres ([1.63 × 10\textsuperscript{6} ± 0.6 × 10\textsuperscript{6}] SD) were injected simultaneously over 30 s, followed by a saline flush. The amount of radiolabeled microspheres given was adjusted to ensure a minimal radioactivity of 0.02 mCi per injection. The order of microsphere injections was randomized in each experiment.

After completion of the study, the animals were given 1,000 U/kg of heparin (Elkkins-Sinn, Cherry Hill, NJ) and 3 mg/kg of papaverine hydrochloride (American Regent Laboratories, Shirley, NY), then exsanguinatated. A midline sternotomy was performed, and catheters were placed in the main pulmonary artery and the left atrium. The lungs were flushed with 50 ml/kg of dextran 2% (Sigma Chemical), removed, inflated to 30 cmH\textsubscript{2}O, and dried with warm air for 7 days. When dry, the lungs were first coated with a one-component polyurethane foam (Kwik Foam, DAP, Dayton, OH), then suspended vertically in a square box and embedded in rapidly setting urethane foam (2 lb. polyol and isocyanate, International Sales, Shirley, NY), then exsanguinated. A midline sternotomy was performed, and catheters were placed in the main pulmonary artery and the left atrium. The lungs were flushed with 50 ml/kg of dextran 2% (Sigma Chemical), removed, inflated to 30 cmH\textsubscript{2}O, and dried with warm air for 7 days. When dry, the lungs were first coated with a one-component polyurethane foam (Kwik Foam, DAP, Dayton, OH), then suspended vertically in a square box and embedded in rapidly setting urethane foam (2 lb. polyol and isocyanate, International Sales, Seattle, WA). The foam block was cut into slices of ~1.2 cm in thickness. With the use of a 12-mm-diameter core, the slices were sampled systematically. Cores were obtained in a rigid X-Y grid system, with 2 cm between the centers of adjacent cores. The height of every core was measured by using a micrometer, and the volume was calculated. Samples [194 ± 39 (SD)] were obtained from each sheep lung samples with airways occupying >50% of the core's volume were discarded.

Twenty-five lung samples were randomly selected from each animal, and the radiolabel-determined and the fluorescent-determined perfusion was obtained. The average volume of the samples was 1.38 ± 0.2 cm\textsuperscript{3}, and the average weight was 0.069 ± 0.02. The radioactivity was measured by using a 3 × 3.25-in. sodium well crystal gamma counter (Minaxi gamma counting system, model 5550, Packard, Downers Grove, IL). Correction for decay time, background counts, and spillover was performed with the matrix inversion method (10). Each tissue piece was counted long enough to ensure a counting error <1%. The samples were then individually soaked for 14 days in 3 ml of 2-ethoxyethanol acetate (Cellosolve, Aldrich Chemical, Milwaukee, WI). The fluorescence was read at days 7, 9, 11, and 14 in a luminescence spectrophotometer (Perkin-Elmer LS-50B, Beaconsfield, Buckinghamshire, UK) fitted with a flow cell and a red-sensitive photomultiplier tube.

The volume-normalized relative blood flow at every time point was calculated for each lung piece, with the color-specific fluorescence and the radiolabel-specific counting performed separately

\[ Q_{rel,i} = \frac{x_i}{(\sum x_i)/n} \]

where \( Q_{rel,i} \) is the volume-normalized relative blood flow of the piece \( i \); \( x_i \) is the obtained fluorescence or counted radioactivity, respectively, divided by the volume of the piece (cm\textsuperscript{3}); and \( n \) is the number of pieces. The mean normalized relative flow was therefore 1.0.

Statistics. The Pearson correlations between the radio- and fluorescent-labeled microsphere relative blood flows were calculated for each animal and each color separately. Correlations were calculated for each color at each soaking time. The mean correlations were compared among colors for a given soaking time and among soaking times for a given color by using the two-tailed paired t-test. A one-sample two-tailed t-test was used to compare correlations to a hypothesized mean of zero. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

The criteria for ARDS were attained after a mean time of 135 ± 65 min after the injection of OA. One animal died 10 min before completion of the study period, and the last injection of microspheres (crimson) could not be given. The total number of coring samples was therefore 150 for crimson and 175 for all other colors.

The mean correlations between the volume-normalized relative blood flows determined by the radiolabeled and the fluorescent microspheres technique were very high and increased only slightly by prolonging the soaking time (Table 1). Statistically significant differences were seen for orange, comparing the 7- and 9-day soaking times, and between orange and blue-green after 7 days of soaking. However, the magnitudes of the differences are extremely minor. Figure 1 shows a representative plot of the radiolabeled-microsphere-determined flow vs. the yellow-green-microsphere-determined flow after 9 days of soaking. In one animal, the correlation for crimson was slightly lower than for other animals: 0.94 after 7 days of soaking, increased to 0.97 after 14 days of soaking. In this animal, the range of relative flow at the time of injection was very small compared with other animals (typical range 0.6 to 1.5). The random measurement error was approximately the same as for other animals, leading to a smaller signal-to-noise ratio and to a slightly smaller correlation.

The accuracy of the fluorescent method is independent of the sample density, as shown by the small and nonsignificant mean correlations between 1) the density of the coring samples and 2) a difference defined by the radiolabeled-microsphere relative flow minus the fluorescent-microsphere relative flow (Table 2). Figure 2 shows a representative plot of the density vs. the radiolabeled-microsphere-determined minus the yellow-
green-microsphere-determined flow after 9 days of soaking, revealing no trends in the difference over the range of the density.

The accuracy of the fluorescent method is also relatively unaffected by the magnitude of flow. Except for blue-green, all the mean correlations between the fluorescence-radioactivity difference with magnitude of flow after 9 days of soaking are nonsignificant and close to zero (Table 3). Bland-Altman plots (5) of fluorescence-radioactive difference as a function of flow after 9 days of soaking show only a slight trend toward greater variability at higher values of relative flow, which is typical of count data. In any case, there is a tight distribution about the mean. Figure 3 shows a representative plot for yellow-green.

**DISCUSSION**

The important finding of this study is that the fluorescent-labeled microspheres technique is an accurate method to measure volume-normalized relative blood flows in severely injured lungs. The linear correlation between perfusion estimates by use of fluorescent and radiolabeled microspheres was excellent. The five chosen colors were easily separable, because each color has a unique and narrow excitation spectrum (7).

![Graph](image)

**Table 1. Mean correlations between radiolabeled vs. fluorescent-microsphere-determined volume-normalized relative flow**

<table>
<thead>
<tr>
<th>Color</th>
<th>Soaking Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 Days</td>
</tr>
<tr>
<td>Red</td>
<td>0.986 ± 0.003</td>
</tr>
<tr>
<td>Orange</td>
<td>0.985 ± 0.004</td>
</tr>
<tr>
<td>Blue-green</td>
<td>0.991 ± 0.002</td>
</tr>
<tr>
<td>Yellow-green</td>
<td>0.988 ± 0.003</td>
</tr>
<tr>
<td>Crimson</td>
<td>0.982 ± 0.008</td>
</tr>
</tbody>
</table>

Values are means ± SE. No. of animals = 7, no. of pieces = 175, for crimson = 150. Significant differences between soaking time: * orange, 9 vs. 7 days, P = 0.03; 11 vs. 7 days, P = 0.03. Significant differences between colors: † orange vs. blue-green at 7 days of soaking, P = 0.03.

The correlation between the two different simultaneously injected microspheres (radioactive and fluorescent) to measure regional perfusion was not perfect. This indicates that there is a measurable, but small, error. The reasons for these errors are well known (9). Several studies have quantified the sources of error for radiolabeled microspheres (1, 6, 13). Glenny et al. (7) suggested that these errors apply also to the fluorescent microsphere technique. They concluded also that the error increases if, in the processing of the lung pieces, there are too many steps involved, leading to a loss of microspheres. They found the best correlations when the samples were only soaked. Therefore, we decided to extract the fluorescent dyes only by increasing the soaking time. We expected a longer soaking time because of the higher density of the tissue compared to noninjured lungs. Noninjured lung samples are usually soaked for 2 days (7). Although the correlations seen after 7 days of soaking were already high, a minor improvement was noted for all colors, and was significant for orange, by increasing the soaking time to 9 days. Further increase in duration of the soaking time showed only a negligible improvement of the correlations.

In most studies investigating pulmonary blood flow, lung pieces with an airway content >25% are discarded, and the relative flow is weight normalized (4, 8, 12). In severely injured lungs, a greater weight range for pieces of the same volume is noted. This is usually due to cellular infiltration or hemorrhage. We volume normalized the relative blood flows to compensate for this variability. We also included pieces up to an airway content of 50% and showed that the accuracy of the

**Table 2. Mean correlation of radiolabeled minus fluorescent microsphere-determined relative flow with magnitude of density after 9 days of soaking**

<table>
<thead>
<tr>
<th>Color</th>
<th>Correlation</th>
<th>P Value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>-0.041 ± 0.098</td>
<td>0.69</td>
<td>-0.232–0.151</td>
</tr>
<tr>
<td>Orange</td>
<td>-0.083 ± 0.091</td>
<td>0.40</td>
<td>-0.262–0.095</td>
</tr>
<tr>
<td>Blue-green</td>
<td>0.062 ± 0.048</td>
<td>0.25</td>
<td>-0.033–0.157</td>
</tr>
<tr>
<td>Yellow-green</td>
<td>-0.015 ± 0.056</td>
<td>0.80</td>
<td>-0.125–0.095</td>
</tr>
<tr>
<td>Crimson</td>
<td>0.112 ± 0.093</td>
<td>0.27</td>
<td>-0.068–0.299</td>
</tr>
</tbody>
</table>

Correlations are means ± SE across animals. All flows were volume normalized. No. of animals = 7, for crimson = 6. No. of pieces = 175, for crimson = 150. Significance was tested against zero with a 2-tailed t-test. CI, confidence interval.
The method is independent of the density of the lung sample. The correlation with the magnitude of density was tested after a soaking time of 9 days because of the results mentioned above.

One important concern when investigating blood flow is that the accuracy of the method should be independent of the magnitude of flow for an individual piece. Whereas the mean correlation for blue-green is significantly different from zero (Table 3), the significance level is not small and could readily have arisen by chance when five hypothesis tests are carried out. In addition, the mean correlation itself is weak. Finally, even if one accepts a real relationship between the fluorescent-isotope difference and the magnitude of flow, the impact is likely to be negligible. The equation for the difference vs. flow indicates a very minor correction: blue-green $-\text{isotope} = 0.033 + 0.033 \times (\text{relative flow})$ (Fig. 4), based on a regression analysis pooling $n = 175$ pieces from seven animals. Across a typical relative flow range of only 0–2.5, the blue-green relative flow would differ from isotope relative flow by $-0.03$ to $+0.05$, an extremely minor correction. The magnitude of this correction is 1–2% of the typical flow range and could be ignored in practice. Even with the use of the equation from the animal with the greatest slope [blue-green $-\text{isotope} = -0.072 + 0.072 \times (\text{relative flow})$], the correction for relative flows from 0 to 2.5 would range from $-0.07$ to $+0.11$, again very minor compared with the wide range of flows encountered.

In conclusion, we have shown that the fluorescent microsphere technique is a valuable tool for investigating regional perfusion in severely injured lungs and can replace radioactive microspheres. The optimal duration of soaking seems to be 9 days, and, if only four colors are used, blue-green can be omitted.

### Table 3. Mean correlation of radiolabeled minus fluorescent microsphere-determined relative flow vs. relative flow after 9 days of soaking

<table>
<thead>
<tr>
<th>Color</th>
<th>Correlation</th>
<th>P Value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>0.124 ± 0.095</td>
<td>0.24</td>
<td>-0.063–0.311</td>
</tr>
<tr>
<td>Orange</td>
<td>-0.022 ± 0.061</td>
<td>0.73</td>
<td>-0.141–0.098</td>
</tr>
<tr>
<td>Blue-green</td>
<td>0.255 ± 0.090*</td>
<td>0.03</td>
<td>0.079–0.432</td>
</tr>
<tr>
<td>Yellow-green</td>
<td>0.083 ± 0.069</td>
<td>0.27</td>
<td>-0.052–0.219</td>
</tr>
<tr>
<td>Crimson</td>
<td>0.175 ± 0.086</td>
<td>0.1</td>
<td>0.006–0.344</td>
</tr>
</tbody>
</table>

Correlations are means ± SE across animals. All flows were volume normalized. No. of animals = 7, for crimson = 6. No. of pieces = 175, for crimson = 150. Significance was tested against zero with a 2-tailed t-test; *P < 0.05. See text for details.
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