Evaluation of Nonradioactive, Colored Microspheres for Measurement of Regional Myocardial Blood Flow in Dogs

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Measurement of regional myocardial blood flow (RMBF) is crucial in experimental studies of myocardial ischemia and reperfusion in dogs. The standard measurement technique uses radioactive microspheres; however, not all institutions are able to dispose of radioactive waste and therefore cannot make use of this method. We tested a new, nonradioactive microsphere, labeled with colors instead of nuclides. Simultaneous blood flow measurements with two nuclide-labeled and two colored microspheres were performed after coronary occlusion in dogs. Both techniques show a within-method correlation of \( r > 0.98 \). Duplicate variability for paired RMBF values in 80 samples was 8.7 ± 0.1% when computed with radioactive microspheres and 13.2 ± 1.8% when computed with colored microspheres. There was a good correlation in the measurement of RMBF between the radioactive- and colored-microsphere methods (\( r = 0.98 \)). The best-fitting linear regression line was expressed by the formula: Colored-microsphere RMBF = 1.11(radiusicro-microsphere RMBF) − 0.02. When measured by colored microspheres, RMBF was approximately 8% higher than when computed with radioactive microspheres for blood flow values of 0–2 ml/min/g. When blood flow was increased pharmacologically to levels of 2–7.5 ml/min/g, colored microspheres yielded blood flow values 39% higher than the values computed by radioactive microspheres. We conclude that the nonradioactive, colored-microsphere method correlates with the radioactive technique, but at high flows, it yields values greater than those obtained with radioactive microspheres. (Circulation 1988;78:428–434)

Use of particles to evaluate flow patterns in the heart was first described in 1909 by Pohlman,1 who injected starch granules into fetal pigs. Since then, many different types of particles have been used to trace flow patterns or measure blood flow in the heart. In 1967, Rudolph and Heymann2 introduced the use of plastic, radioactive microspheres for measurement of regional perfusion, and Makowski et al3 later added the concept of the reference sample, which made it possible to calculate blood flow. Use of radioactive microspheres for the measurement of regional myocardial blood flow (RMBF) in dogs was first validated by Domenech et al.4 The radioactive-microsphere method is relatively simple, and its validity is well documented.5–8 The technique has become standard for the measurement of RMBF in experimental cardiology. However, there are disadvantages such as the high cost of the multichannel gamma analyzer required by the technique and, of primary consideration in this era of ecological and cost consciousness, the expense and inconvenience of the disposal of radioactive carcasses.

A new type of microsphere has recently been described by Shell and coworkers.9 These microspheres are nonradioactive and are labeled with various colors. They are used in a manner identical to the radioactive microspheres; however, instead of analyzing the samples for radioactive counts, the samples are digested, and the actual number of microspheres is counted. Our objective was to evaluate this colored-microsphere method and compare it with the radioactive-microsphere technique in a canine model.

Materials and Methods

Microsphere Preparation

Radioactive, polystyrene microspheres, 11.3 ± 0.1 \( \mu \text{m} \) (mean ± SD), labeled with \(^{89}\text{Nb}, ^{141}\text{Ce}, \) or \(^{103}\text{Ru} \) were obtained from DuPont Research Products, Billerica, Massachusetts. The stock solution (micro-
spheres in 10% dextran with 0.01% Tween 80) was agitated, sonicated for 30 minutes, and was then diluted in sterile 53% sucrose to obtain a solution containing $5 \times 10^3$ spheres/ml. Before use, the microsphere solution was brought to room temperature and placed on a rotator to keep the microspheres dispersed.

Colored, polystyrene microspheres, $11.9 \pm 1.9 \mu m$, were obtained from E-Z Trac, Los Angeles, California. These microspheres are available with nine different color labels. In this study, orange, yellow, and blue were used. Vials obtained from the manufacturer contained approximately $1 \times 10^6$ microspheres/ml. Samples from each vial of stock solution (microspheres in water containing 0.025% Tween 80 and 1% thimerosal) were diluted and counted with a hemocytometer to determine the actual number of spheres per milliliter in each vial. When viewed in a hemocytometer at $\times 200$, each color is visually distinct; thus, it is possible to accurately count small numbers of one colored microsphere when large numbers of another are present. For each experiment, the vials were manually agitated and vortex mixed for 1 minute; then, an aliquot containing approximately $6 \times 10^6$ microspheres was removed from the vials and placed in a small nonwetting plastic container. We chose to give a relatively high number of colored microspheres per measurement to improve the level of precision as the samples were counted manually in a hemocytometer, and error is reduced when larger numbers of microspheres are counted. Immediately before injection, the radioactive microsphere solutions were added to the container, and the microspheres were dispersed and mixed by drawing the solution back and forth through an 18-gauge needle into a 10-ml syringe. When examined microscopically, both radioactive and colored microspheres appeared spherical, and neither showed evidence of aggregation. As indicated by the standard deviation of the diameters, colored microspheres appeared to have greater variation in size.

Animal Preparation

Animals used in this study were maintained in accordance with the guidelines of the Division of Laboratory Animal Resources of Wayne State University and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. 85-23, revised 1985).

Mongrel dogs ($n=10$) of either sex were anesthetized with sodium pentobarbital (35 mg/kg i.v.) and mechanically ventilated with room air. Additional anesthesia was administered when required. Fluid-filled catheters were placed into the left jugular vein for administration of fluids and into the left carotid and femoral arteries for measurement of systemic arterial pressure and withdrawal of reference blood samples. The chest was opened at the fifth left intercostal space, and the pericardium was incised. A catheter was placed into the left atrium through the left atrial appendage for the administration of microspheres. In Protocol 1, the left anterior descending coronary artery was isolated proximal to the first diagonal branch for later occlusion. Heart rate and systemic arterial pressure were monitored throughout the duration of the experiment.

Protocol 1 ($n=8$). After a short period of stabilization (5–10 minutes) and lidocaine administration (1.5 mg/kg) in some dogs ($n=4$), the left anterior descending coronary artery was occluded. Five minutes after occlusion, a blood-flow measurement was performed with two colored microspheres ($6 \times 10^6$ of each) and two nuclide-labeled microspheres ($2 \times 10^6$ of each) injected simultaneously. A reference blood sample was obtained from the femoral artery at the rate of 15.3 ml/min starting 5 seconds before the injection and continuing until 60 seconds after the injection (total collection time, $\sim 90$ seconds). The number of microspheres injected and the sample withdrawal rate were chosen to yield a large number of microspheres in reference and tissue samples. This helps minimize statistical fluctuations due to random distribution of microspheres. In six animals, a second reference sample was obtained from the carotid artery with the same withdrawal pump. After completion of the flow measurement, Monastral blue dye was injected into the left atrium to delineate the in vivo risk zone of ischemia; then, the dog was killed with potassium chloride injected through the left atrial catheter. The heart was excised; the left ventricle was sliced transversely into 1.5-cm sections, and 10 transmural samples (2–3 g each) from a midventricular slice were obtained from each dog. Samples included zones of ischemic, intermediate, and normal blood flow as indicated by the Monastral dye. The zone of intermediate flow was tissue that straddled the Monastral blue line.

Protocol 2 ($n=2$). In two dogs, we performed three repeated measurements, pharmacologically varying myocardial blood flow levels between measurements. A baseline RMBF measurement was obtained with a randomly selected nuclide-labeled microsphere and a randomly selected colored microsphere. After the baseline measurement, epinephrine (3 $\mu g/kg$) was administered to elevate blood flow, and a second measurement was made with a different nuclide and color. After 15 minutes, a large dose of an ultra-short acting $\beta$-blocker (Esmonol, 0.6–1.5 mg/kg) was given to decrease blood flow. A third measurement was performed with a third nuclide and a third color. After death, the heart was excised, and 10 transmural samples (2–3 g) were obtained from each left ventricle.

Sample Processing

All reagents used for the processing of blood and tissue samples to quantify colored microspheres
were obtained from E-Z Trac; the manufacturer’s names for the various reagents will be used.

Blood. The reference blood samples, together with saline washings of the syringes, were placed into conical plastic tubes and centrifuged. After measuring radioactivity, the blood was again centrifuged for 15 minutes and the plasma aspirated. The red cell sediment was diluted to 40 ml with Blood Hemolysis Reagent and centrifuged for 30 minutes. The supernatant was aspirated, and 25 ml distilled water was added to the sediment. This was mixed and centrifuged for 15 minutes, and the supernatant was again aspirated. Blood Digest Reagent was then added to the sediment, and the sample was placed in boiling water for 30 minutes. After cooling, the digest was diluted with 25 ml distilled water, and the sample was centrifuged for 15 minutes. The supernatant was aspirated, and 4 ml Blood Microsphere Counting Reagent was added to the sediment. After vortex mixing, the mixture was transferred to a smaller tube; the larger tube was washed three times with 2 ml Blood Microsphere Counting Reagent, and the washes were added to the smaller tube. The samples were again centrifuged for 15 minutes, and then the supernatant was aspirated leaving 0.2–0.4 ml of the supernatant in the bottom of the tube. After vortex mixing, the volume was measured by drawing the mixture up into the tip of a variable-volume automatic pipette. This volume is the final dilution volume of the microspheres.

Tissue. After weighing, the tissue samples were minced finely with a scalpel, were placed in tubes, and were measured for radioactivity. The manufacturer of the colored microspheres recommended a sample of 2–3 g. In a pilot study, we counted the 2–3-g sample in a single tube. Our results indicated that some quenching of counts in the large sample occurred. Therefore, in this study, each sample was subdivided into three pieces weighing 0.8–1 g, the sample size normally used in this laboratory for the measurement of radioactivity. The three pieces were counted individually, and then the counts were pooled for each sample. The sample was added to a 50-ml tube together with 15 ml Tissue Digest Reagent I and placed in a boiling water bath for 1 hour with a short period of cooling and a 1-minute vortex mixing after 30 minutes. The sample was then diluted to 40 ml with Tissue Digest Reagent II and centrifuged for 30 minutes. The supernatant was poured off, and the sample was resuspended in 4 ml Tissue Microsphere Counting Reagent. After vortex mixing, the mixture was transferred to a smaller tube, and the larger tube was washed three times with 2 ml Tissue Microsphere Counting Reagent. The samples were again centrifuged for 15 minutes, and the supernatant was then aspirated, leaving 0.2–0.4 ml of the supernatant in the bottom of the tube. After vortex mixing, the final dilution volume was measured by drawing up the mixture into the tip of a variable-volume automatic pipette.

**FIGURE 1.** Regression plots of paired within-method regional myocardial blood flow (RMBF, ml/min/g). Panel A: Paired radioactive microspheres, Nb=0.93(Ce)−0: r=0.998; mean percent difference between pairs is 8.7±0.1%. Panel B: Paired colored microspheres, yellow=1.09(blue)−0.02; r=0.986; mean percent difference between pairs is 13.2±1.8%. Each point represents one tissue sample.

**Microsphere Counting and Calculations**

Radioactive microspheres. Samples were counted for 2.5 minutes with a multichannel pulse-height analyzer (model ND62, Nuclear Data, Schaumburg, Illinois). After appropriate correction for background and crossover, computed by spectral stripping,10 RMBF was computed in each sample with the formula: RMBF=(CT×R)/(CR×WT), where CT is counts in the tissue sample, R is reference flow rate (ml/min), CR is counts in the reference blood sample, and WT is weight of the tissue sample in grams. Results are expressed as milliliters per minute per gram.

Colored microspheres. Aliquots of the final solution were placed in an Improved Neubauer Hemocytometer. Twelve chambers were counted for each sample, and the total number of microspheres in each reference blood and tissue sample was computed with the formula:

Total microspheres =

$$\frac{\text{No. counted} \times 1,000 \text{ mm}^3}{\text{No. chambers} \times 0.9 \text{ mm}^2 \times \frac{1}{\text{ml}}} \times \text{ml suspension}$$
where No. counted is the number of colored microspheres counted, No. chambers is the number of chambers counted, 0.9 mm$^3$ is the ruled volume of the chamber, and ml suspension is the final dilution volume. RMBF was then computed with the same formula that was used for calculating radioactive microspheres. RMBF = (CT × R)/(CR × WT), where CT is the total number of microspheres in the tissue sample, R is reference flow rate (ml/min), CR is the total number of microspheres in the reference blood sample, and WT is weight of the tissue sample in grams. Results are expressed as milliliters per minute per gram.

**Statistical Analyses**

Analyses were performed within and between methods by linear regression and paired $t$ test. Analysis of variance (ANOVA) was used to compare values obtained by the four microspheres in the three zones of flow (ischemic, intermediate, and normal). Data are expressed as mean ± SEM.

**Results**

**Protocol 1**

Heart rate and mean arterial blood pressure were similar before and after the blood flow measurements. One minute before the blood flow measurement, mean heart rate in all animals was 144 ± 7 beats/min, and after completion of the measurement, it was 153 ± 5 beats/min ($p = NS$). Mean arterial blood pressure was 105 ± 9 mm Hg before measurement and 99 ± 12 mm Hg after ($p = NS$). Thus, the microsphere injection itself did not alter systemic hemodynamics.

The simultaneous injection of two of each type of microsphere allows comparisons both within and between the two methods. Figure 1 shows plots of paired RMBF measurements calculated with $^{95}$Nb and $^{141}$Ce microspheres and paired RMBF measurements calculated with yellow and blue microspheres in the 80 samples. Between $^{95}$Nb and $^{141}$Ce microspheres, the correlation coefficient was 0.998, and the duplicate variability (the absolute value of the difference of each pair divided by each arithmetic mean $\times 100$) was $8.7 \pm 0.1\%$. The SEE was 0.03 ml/min/g. There was also an excellent correlation between yellow and blue microsphere pairs ($r = 0.986$). However, with yellow and blue microspheres, there was greater scatter. The duplicate variability was $13.2 \pm 1.8\%$, and the SEE was 0.09 ml/min/g.

Individual $^{95}$Nb- and $^{141}$Ce-microsphere values of RMBF are plotted against individual yellow- and blue-microsphere values of RMBF in all tissue samples in Figure 2. Slopes of the best-fitting regression line ranged from 1.02 to 1.20. Correlation coefficients for all paired comparisons exceeded 0.97. Because RMBF calculations for all tissue samples in each dog depend on the value of a single reference blood sample, and thus are not independent, we calculated the means of all 10 samples in each dog to obtain a single value for each of the four microspheres. Table 1 shows the results of linear regression analysis for the mean of 10 samples in the eight dogs. Correlation coefficients for all com-
TABLE 1. Linear Regression Analysis Among Microspheres

<table>
<thead>
<tr>
<th>Microspheres</th>
<th>Slope</th>
<th>Intercept</th>
<th>r</th>
<th>SEE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow vs. ^9^Nb</td>
<td>1.35*</td>
<td>-0.11</td>
<td>0.98</td>
<td>0.07</td>
</tr>
<tr>
<td>Blue vs. ^9^Nb</td>
<td>1.14</td>
<td>-0.03</td>
<td>0.98</td>
<td>0.06</td>
</tr>
<tr>
<td>Yellow vs. ^14^Ce</td>
<td>1.27*</td>
<td>-0.12</td>
<td>0.98</td>
<td>0.07</td>
</tr>
<tr>
<td>Blue vs. ^14^Ce</td>
<td>1.08</td>
<td>-0.04</td>
<td>0.98</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*Slope differs from line of identity, p<0.05. Intercepts were not significantly different from 0.

Comparisons exceeded 0.98, and the SEE ranged from 0.06 to 0.07 ml/min/g.

We examined blood flow values obtained with the four microspheres in the three regions that we sampled. The mean RMBF calculated with ^9^Nb, ^14^Ce, and yellow and blue microspheres in zones of normal, intermediate, and low blood flow are shown in Table 2. There were no significant differences between these means.

To make comparisons between methods, we used the mean of ^9^Nb and ^14^Ce RMBF in each sample as an estimate of “radioactive” RMBF (RM-RMBF) and the mean of yellow and blue RMBF in each sample as an estimate of “colored” RMBF (CM-RMBF). Figure 3 is a plot of the 80 samples and the best-fitting regression line expressed by the formula: CM-RMBF = 1.11(RM-RMBF) - 0.02. The between-method correlation coefficient was 0.98. On average, in this protocol with a range of flows from 0.02 to 1.96 ml/min/g, colored microspheres yielded RMBF values 8±2% higher than radioactive microspheres (p<0.002).

We examined the variation in paired reference samples obtained from the carotid and femoral arteries in six dogs. The variability obtained in paired reference samples is similar to that sometimes found in a given organ. First, the total number of radioactive counts was measured in each reference sample, and then the total number of colored microspheres was computed. When expressed as the absolute value of the difference of carotid and femoral values divided by their mean, the following percent differences were observed: ^9^Nb, 3.1±1.1%; ^14^Ce, 2.8±0.9%; yellow, 4.7±1.4%; and blue, 5.7±2.0%. The average variation for radioactive microspheres was 3.0±1.0% and for colored microspheres was 5.2±1.5%. Thus, variability of microsphere counts was comparable in reference samples from the carotid and femoral arteries for both radioactive and colored microspheres.

Protocol 2

RMBF measurements were made with paired colored and radioactive microspheres during baseline and high- and low-flow conditions in two animals. Table 3 shows the results of these two experiments. Each flow represents the mean±SEM of 10 samples obtained from each animal. Figure 4 is a plot of all (10 samples, three flows) radioactive and colored RMBF measurements from the two dogs. The best-fitting line of regression is expressed by the formula CM-RMBF = 1.35(RM-RMBF) - 0.13, r = 0.99. In this protocol, RMBF values obtained with colored microspheres were on average 8±3% higher for baseline and low-flow conditions with RMBF values of 0.24-1.10 ml/min/g but were 39±4% higher for blood flow above 2 ml/min/g.

Discussion

Since its introduction in the 1960s, the radioactive-microsphere technique has been the "gold standard" for measurement of regional blood flow in
studies of experimental myocardial ischemia in dogs. The method is simple and reliable; however, its use is limited to institutions that have methods for disposal of radioactive waste. In the present study, we examined an alternative technique in which microspheres were labeled with various colors instead of radioactive nuclides. This technique allows measurement of regional blood flow in a manner identical to that of radioactive microspheres, but it obviates the need for time-consuming record keeping and expensive disposal of radioactive materials.

Injection of two nuclide-labeled and two colored microspheres simultaneously allowed us to make direct comparisons within and between the two techniques of measuring regional blood flow. In this study, duplicate variability computed between the two radioactive microspheres was 8.7 ± 0.1%. This is similar to that reported by Bassingthwaighte et al. With colored microspheres, duplicate variability was greater (13.2 ± 1.8%).

In both protocols, there was an excellent correlation between the two techniques (>0.98). For RMBF values in the normal physiological range in the dog, up to 2 ml/min/g, blood flow computed with colored microspheres was approximately 8% higher than that computed with radioactive microspheres. When RMBF was pharmacologically augmented with epinephrine to achieve levels of 2.0–7.5 ml/min/g, blood flow values were on average 39% higher when computed with colored microspheres.

The discrepancy in blood flow values at high-flow levels may be due to differences in the microspheres themselves. Although both microspheres are made of polystyrene, the density of radioactive microspheres is 1.4 g/ml. Colored microspheres have a density of 1.05 g/ml, which is closer to that of red cells (1.098 g/ml), and they have greater variation in diameter than radioactive microspheres. When 1,070 colored microspheres were analyzed, the range of diameters was 3.3–29.2 µm, with 98% having diameters between 8.1 and 15.7 µm (personal communication from E-Z Trac). For radioactive microspheres, 98% of the diameters are between 11.1 and 11.5 µm. However, Utley et al. showed that there was less than 1% difference between the distribution of 15-µm microspheres and 9-µm microspheres to the left ventricle.

The colored-microsphere technique has several advantages. Special disposal of carcasses is not necessary because the microspheres are not radioactive. It is unnecessary to purchase a major, expensive piece of equipment, for example, a multichannel gamma analyzer. The microspheres are counted directly in the tissue so that there are no problems with quenching of counts due to sample geometry, and crossover errors are eliminated. With radioactive microspheres, there is often waste due to radioactive decay of short half-life isotopes; colored microspheres are stable in vitro for up to 1 year and in vivo for at least 48 hours, according to the manufacturer. Potential disadvantages include the finding that in the limited number of animals in this study in which high blood flow was induced, the colored-microsphere method yielded values higher than radioactive microspheres. However, it cannot be determined from this data whether colored microspheres overestimate true flow or whether radioactive microspheres underestimate flow. Also, data obtained with colored microspheres showed slightly more scatter. Another disadvantage of using colored microspheres is the current counting method (hemocytometer), which requires considerable time and is tedious. Presently, it takes approximately 45 minutes to process each sample compared with 5 minutes for each sample when RMBF is measured with radioactive microspheres. However, future developments of automated sample processing and microsphere counting would resolve this problem.

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**KEY WORDS** • regional myocardial blood flow • radioactive microspheres • colored microspheres
Evaluation of nonradioactive, colored microspheres for measurement of regional myocardial blood flow in dogs.
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Circulation. 1988;78:428-434
doi: 10.1161/01.CIR.78.2.428

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1988 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

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