Validity of fluorescent microspheres method for bone blood flow measurement during intentional arterial hypotension

H. Anetzberger,1,2 E. Thein,2 M. Becker,1,2,3 A. K. Walli,3 and K. Messmer2
1Department of Orthopaedics, 2Institute for Surgical Research, and 3Institute for Clinical Chemistry, Klinikum Grosshadern, Ludwig-Maximilians University, 81377 Munich, Germany
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Anetzberger, H., E. Thein, M. Becker, A. K. Walli, and K. Messmer. Validity of fluorescent microspheres method for bone blood flow measurement during intentional arterial hypotension. J Appl Physiol 95: 1153–1158, 2003. First published May 2, 2003; 10.1152/japplphysiol.00299.2003.—In this study, we compared bone blood flow values obtained by simultaneously injected fluorescent (FM) and radiolabeled microspheres (RM) at stepwise reduced arterial blood pressure. Ten anesthetized female New Zealand White rabbits received simultaneous left ventricular injections of FM and RM at 90, 70, and 50 mmHg mean arterial blood pressure (MAP). After the experiments, both kidneys and long bones of all four limbs were removed and dissected in a standardized manner. Radioactivity (corrected for decay, background, and spillover) and fluorescence were determined, and blood flow values were calculated. Relative blood flow values estimated for each bone sample by RM and FM were significantly correlated (r = 0.98, slope = 0.99, and intercept = 0.04 for 90 mmHg; r = 0.98, slope = 0.94, and intercept = 0.09 for 70 mmHg; r = 0.98, slope = 0.96, and intercept = 0.07 for 50 mmHg). Blood flow values (ml·min⁻¹·100 g⁻¹) of right and left bone samples determined at the different arterial blood pressures were identical. During moderate hypotension (70 mmHg MAP), blood flow in all bone samples remained unchanged compared with 90 mmHg MAP, whereas a significant decrease of bone blood flow was observed at severe hypotension (50 mmHg MAP). Our results demonstrate that the FM technique is valid for measuring bone blood flow. Differences in bone blood flow during altered hemodynamic conditions can be detected reliably. In addition, changes in bone blood flow during hypotension indicate that vasomotor control mechanisms, as well as cardiac output, play a role in setting bone blood flow.

The microsphere method for determination of regional bone blood flow (RBBF) is well established (12–14, 19, 22, 23, 25, 27). The advantage of using microspheres to determine RBBF is obvious, because it is possible to carry out repetitive measurements without surgical manipulation of the bone. Microparticles such as radioactive microspheres (RM) (12, 13, 19, 22, 23, 25–27), resin particles (6), colored microspheres (CM) (20, 39, 45), and fluorescent microspheres (FM) (29) are suitable for use. To obtain valid and precise data using the microsphere method, sources of error must be taken into account. Most of the errors evaluated with RM can be related to nonradioactive spheres as well. The stochastic error due to the Poisson nature of the sphere distribution is minimized by injection of a sufficient number of spheres. Methodological errors due to reference sample collection or inadequate mixing of microspheres in the blood, nonentrapment, shunting, and aggregation of the spheres are independent of the sphere species and can be avoided by an appropriate experimental setup. Because the use of RM for measurement of RBBF is limited in long-term studies (44) and problematic because of the health hazards and safety concerns, CM or FM were introduced. However, in contrast to RM, methodological errors because of loss of dye or spheres during sample processing may occur with CM or RM. Recently developed methods that employ a sample-processing unit (SPU) and an automated sample-processing technique minimize this problem, particularly for FM (32, 43). Compared with CM, a further advantage of using FM is the proven applicability for chronic in vivo studies (44).

Since the validation of FM by Glenny et al. (16), this technique has been used for various organs (16, 31, 34, 35, 40, 42), except bone, which is a compact tissue and presents various sample-processing problems. Some authors tried to quantify the number of FM by fluorescence microscopy (29, 39) or by extraction of the fluorescent dye from the FM without prior digestion of the bone (41). However, these techniques do not ensure quantitative recovery of dyes from the microspheres. In a recent publication, we demonstrated that bone digestion by means of HCl does not influence the fluorescent characteristics of the spheres (2).

Nevertheless, the validity of measuring bone blood flow with the FM technique has not been demonstrated. Therefore, the aim of this study was to compare RBBF data obtained by injecting pairs of FM and RM simultaneously during graded arterial hypotension.

MATERIALS AND METHODS

Ten adult female New Zealand White rabbits (Charles River, Kisslegg, Germany) with a mean body weight of 3.0 ±
Correct position of the catheter cannulated with a catheter passed into the left ventricle. The right common carotid artery was isolated and a total volume of 10.1 ml. For analysis of cardiac output (CO), Table 2.

Regression analysis and difference of RBBF determined by RM and FM

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MAP</th>
<th>n</th>
<th>r</th>
<th>r²</th>
<th>Slope</th>
<th>Intercept</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90 mmHg</td>
<td>2</td>
<td>0.9</td>
<td>0.96</td>
<td>0.99 (0.97–1.01)</td>
<td>0.04 (0.02–0.07)</td>
<td>0.03 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>70 mmHg</td>
<td>2</td>
<td>0.9</td>
<td>0.96</td>
<td>0.94 (0.92–0.96)</td>
<td>0.09 (0.07–0.12)</td>
<td>0.03 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>50 mmHg</td>
<td>2</td>
<td>0.9</td>
<td>0.95</td>
<td>0.96 (0.97–0.99)</td>
<td>0.07 (0.04–0.09)</td>
<td>0.03 ± 0.16</td>
</tr>
</tbody>
</table>

Values for difference are means ± SD. Values in parentheses are 95% confidence intervals. RBBF, regional bone blood flow; RM and FM, radioactive and fluorescent microspheres; n, no. of samples; r, correlation coefficient; r², coefficient of determination.

BLOOD FLOW MEASUREMENT

Table 1. Physiological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MAP, mmHg</th>
<th>90 mmHg</th>
<th>70 mmHg</th>
<th>50 mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, min⁻¹</td>
<td>167 ± 25</td>
<td>156 ± 23</td>
<td>163 ± 23</td>
<td></td>
</tr>
<tr>
<td>CO, ml · min⁻¹ · kg⁻¹</td>
<td>120 ± 34</td>
<td>119 ± 11</td>
<td>120 ± 8</td>
<td></td>
</tr>
<tr>
<td>PaO₂, Torr</td>
<td>42 ± 5</td>
<td>39 ± 5</td>
<td>38 ± 3</td>
<td></td>
</tr>
<tr>
<td>Arterial pH</td>
<td>7.40 ± 0.04</td>
<td>7.43 ± 0.05</td>
<td>7.43 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>7.6 ± 1.4</td>
<td>7.2 ± 1.2</td>
<td>7.0 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Hct, %</td>
<td>22 ± 4</td>
<td>21 ± 4</td>
<td>20 ± 4</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 10. MAP, mean arterial blood pressure; CO, cardiac output; PaCO₂ and PaO₂, arterial partial pressure of CO₂ and O₂, respectively. *Significantly different from 90 mmHg (P < 0.05); †significantly different from 70 mmHg (P < 0.05).

0.1 kg were used in this study, which was approved by the Animal Care Committee at the Bavarian State Authorities. The animals were anesthetized by an intramuscular injection of ketamine (15 mg/kg body wt) and xylazine (2 mg/kg body wt) and fixed in the supine position. Anesthesia was maintained by continuous intravenous infusion of ketamine (10 mg·kg body wt⁻¹·h⁻¹) and xylazine (2.4 mg·kg body wt⁻¹·h⁻¹). The animals were intubated and mechanically ventilated. The right common carotid artery was isolated and cannulated with a catheter passed into the left ventricle. Correct position of the catheter’s tip was confirmed by the typical waveform of the left ventricular pressure curve. The catheter served for the injection of microspheres. A second catheter for collection of the arterial reference blood sample and for measurement of arterial blood pressure was introduced into the left carotid artery and advanced into the descending aorta. Blood pressure and heart rate were continuously monitored throughout the experiment. Mean arterial blood pressure (MAP) was adjusted at 90, 70, or 50 mmHg by intravenous infusion of 6% Haemofusin (Baxter, Unterschleissheim, Germany) or withdrawal of venous blood and maintained at the predetermined level for 20 min until the injection of microspheres. Before each injection, arterial PO₂, PaCO₂, and pH were determined.

Bone blood flow measurements. Six differently labeled FM (FluoSpheres, Molecular Probes, Eugene, OR; 15.5 ± 0.3 μm diameter) and RM labeled with ⁵¹Cr, ¹⁴₁Ce, and ⁴⁶Sc (NEN-TRAC, NEN Life Science Products, Boston, MA; 15.5 ± 0.1 μm diameter) were used. Both microsphere techniques are routine in our institute; our experimental techniques for measurement of regional blood flow of solid organs and bone have been described in detail (2, 21, 32, 43).

To test the methodological variability, the RM and FM injections were carried out simultaneously. The species of microspheres were selected randomly in each experiment. Before each injection, the microspheres were vortexed and sonicated. Approximately 3 × 10⁶ RM and FM each were mixed together in a syringe and suspended with 0.9% NaCl to a total volume of 10.1 ml. For analysis of cardiac output (CO), radioactivity and fluorescence were measured in 20 μl of this suspension. Microspheres were injected over a period of 1 min. At 15 s before the injection, withdrawal of an arterial reference blood sample was started by means of a Harvard pump (model 33 syringe pump, FMI, Egelsbach, Germany). Reference blood sampling was performed for 2 min at a constant withdrawal rate of 3.54 ml/min.

After the last injection of microspheres, the animals were killed with an overdose of pentobarbital sodium. Both kidneys and humerus, femur, and tibia bones were sampled. Each kidney was dissected according to a hierarchical scheme into eight samples. Muscles, periosteum, and ligaments, as well as cartilage, were removed from the long bones. Each femur, tibia, and humerus was dissected according to a constant scheme into eight, seven, and five bone samples, respectively. The tissue samples were weighed immediately after dissection.

Radioactivity of each tissue and reference blood sample was determined for 3 min by means of a gamma counter (Auto-Gamma 5650, Canberra Packard, Frankfurt/Main, Germany). Each sample was corrected for decay time, background counts, and spillover by means of a matrix inversion method using the MIC III system (21).

Fluorescence of reference blood and kidney samples was measured directly after the radioactivity was counted. Fluorescence of the bone samples was measured after the crystalline matrix was dissolved for 3 wk in HCl (1 mol/l) under protection from light. The further sample-processing steps, including sample digestion, isolation of FM, and on-line measurement of fluorescence using a luminescence spectrometer (model LS50B, Perkin-Elmer, Ueberlingen, Germany), were carried out by means of our automated method (43) based on the SPU (Gaiser, Kappel-Grafenhausen, Germany) (32).

The data originating from the radioactivity and fluorescence measurements were used to calculate the blood flow values (ml·min⁻¹·100 g⁻¹) for each tissue sample and for each injection time point according to the following formula

\[ Q_{\text{sample}} = Q_{\text{ref}} \times I_{\text{sample}} \times I_{\text{ref}}^{-1} \]

where \( Q_{\text{sample}} \) is blood flow in the sample (ml/min), \( Q_{\text{ref}} \) is withdrawal rate of the Harvard pump (3.54 ml/min), \( I_{\text{sample}} \) is fluorescent intensity/radioactivity of the sample, and \( I_{\text{ref}} \) is fluorescent intensity/radioactivity of the reference blood sample. \( Q_{\text{sample}} \) was then divided by the tissue weight and normalized to 100 g.

Peripheral vascular resistance was calculated by dividing the MAP (mmHg) by the blood flow (ml·min⁻¹·100 g⁻¹) of each sample.

CO was determined as follows

\[ CO = Q_{\text{ref}} \times I_{\text{inj}} \times I_{\text{ref}}^{-1} \times BW^{-1} \]

where CO is cardiac output (ml/min), \( Q_{\text{ref}} \) is flow rate of the Harvard pump (3.54 ml/min), \( I_{\text{inj}} \) is fluorescence intensity/radioactivity injected (\( I_{\text{inj}} = 500 \times I_{20 \mu l} \)), and \( I_{20 \mu l} \) is the fluorescence intensity of 20 μl of the injected dose, \( I_{\text{ref}} \) is fluorescent intensity/radioactivity of the reference blood sample, and BW is body weight.

Table 2. Regression analysis and difference of RBBF determined by RM and FM

<table>
<thead>
<tr>
<th>MAP, mmHg</th>
<th>n</th>
<th>r</th>
<th>r²</th>
<th>Slope</th>
<th>Intercept</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>371</td>
<td>0.9</td>
<td>0.96</td>
<td>0.99 (0.97–1.01)</td>
<td>0.04 (0.02–0.07)</td>
<td>0.03 ± 0.14</td>
</tr>
<tr>
<td>70</td>
<td>372</td>
<td>0.98</td>
<td>0.96</td>
<td>0.94 (0.92–0.96)</td>
<td>0.09 (0.07–0.12)</td>
<td>0.03 ± 0.15</td>
</tr>
<tr>
<td>50</td>
<td>372</td>
<td>0.98</td>
<td>0.95</td>
<td>0.96 (0.97–0.99)</td>
<td>0.07 (0.04–0.09)</td>
<td>0.03 ± 0.16</td>
</tr>
</tbody>
</table>

Values for difference are means ± SD. Values in parentheses are 95% confidence intervals. RBBF, regional bone blood flow; RM and FM, radioactive and fluorescent microspheres; n, no. of samples; r, correlation coefficient; r², coefficient of determination.
RESULTS

Physiological parameters. All measurements were performed under standardized conditions. Before the first injection of MS, MAP was adjusted to 90 mmHg by intravenous infusion of 6% Haemofusin solution (40 ± 33 ml). MAP was lowered to 70 mmHg by withdrawal of 24 ± 15 ml of blood, and a further reduction of MAP to 50 mmHg was achieved by withdrawal of an additional 10 ± 7 ml of blood. Arterial blood gases remained constant throughout the experiment (Table 1). CO remained unchanged at 90 and 70 mmHg MAP but was significantly reduced by ~30% at 50 mmHg MAP.

Relative bone blood flow. To determine differences between the two methods, all relative bone blood flow values obtained by simultaneously injected FM and RM at 90, 70, and 50 mmHg MAP were compared using least squares linear regression and the method of Bland and Altman (5). The regression lines were as follows: $y = 0.99x + 0.04 \ (r^2 = 0.96)$ at 90 mmHg MAP, $y = 0.94x + 0.09 \ (r^2 = 0.96)$ at 70 mmHg MAP, and $y = 0.96x + 0.07 \ (r^2 = 0.95)$ at 50 mmHg MAP. The confidence intervals of the slope were close to 1, and the confidence intervals of the intercept were close to zero (Table 2). The mean difference of the relative bone flow values obtained by RM and FM at 90, 70, and 50 mmHg MAP was close to zero and showed a uniform distribution of scatter above and below zero. The linear regression analysis and the comparison by the method of Bland and Altman for all bone samples at 90 mmHg MAP are presented in Fig. 1.

Table 3. Blood flow values and vascular resistance for each organ

<table>
<thead>
<tr>
<th>Sample</th>
<th>Blood Flow, ml·min⁻¹·100 g⁻¹</th>
<th>Vascular Resistance, mmHg · ml⁻¹ · min⁻¹ · 100 g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90 mmHg</td>
<td>70 mmHg</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>298 ± 47</td>
<td>326 ± 53</td>
</tr>
<tr>
<td>Left</td>
<td>284 ± 46</td>
<td>310 ± 51</td>
</tr>
<tr>
<td>Femur</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>10.9 ± 1.2</td>
<td>11.9 ± 1.6</td>
</tr>
<tr>
<td>Left</td>
<td>10.9 ± 1.1</td>
<td>11.8 ± 1.4</td>
</tr>
<tr>
<td>Tibia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>6.8 ± 0.8</td>
<td>6.6 ± 0.8</td>
</tr>
<tr>
<td>Left</td>
<td>6.7 ± 0.8</td>
<td>6.5 ± 0.8</td>
</tr>
<tr>
<td>Humerus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>13.6 ± 1.3</td>
<td>14.7 ± 1.4</td>
</tr>
<tr>
<td>Left</td>
<td>13.3 ± 1.4</td>
<td>14.5 ± 1.7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10. *Significantly different from 90 mmHg (P < 0.05); †significantly different from 70 mmHg (P < 0.05).
Regional blood flow. Blood flow data were obtained by means of RM, because radioactivity could be analyzed in all samples, whereas fluorescence could be analyzed in 373 (93%) of 400 bone samples; 27 samples were lost during sample processing using the automated procedure. Four single measurement values were lost because of technical problems with the luminescence spectrometer.

The weights of right and left kidneys (7.2 ± 0.3 and 7.6 ± 0.3 g, respectively) and femur (10.0 ± 0.3 and 10.0 ± 0.3 g, respectively), tibia (8.1 ± 0.2 and 8.0 ± 0.2 g, respectively), and humerus (4.9 ± 0.1 and 4.9 ± 0.1 g, respectively) bones were comparable.

Blood flow values (ml·min⁻¹·100 g⁻¹) did not differ at any time of comparison of all right and left long bones and both kidneys (Table 3). Mean bone blood flow was significantly higher in humerus than in femur and tibia. Mean blood flow in both kidneys did not differ at 90 and 70 mmHg MAP. At 50 mmHg MAP, blood flow significantly decreased in kidneys by 27%, in humerus by 18%, in femur by 21%, and in tibia by 29%. Corresponding findings were observed in all other bone samples (Fig. 2).

The vascular resistance in all organs and within each tissue sample was significantly lower at 70 than at 90 mmHg MAP. No further change of vascular resistance was observed when MAP was lowered from 70 to 50 mmHg (Table 3).

DISCUSSION

Our data show that measurement of RBBF by means of FM is an accurate and reliable alternative to the RM method. Differences in RBBF due to altered hemodynamic conditions can be detected. Furthermore, we could show that the mean blood flow was higher in the humerus than in the femur and tibia. Within the long bones, we found a heterogeneity of blood perfusion.

Under moderate hypotension (70 mmHg MAP), blood flow remained unaffected, whereas a significant decrease of RBBF was observed at severe hypotension (50 mmHg MAP).

The advantages of FM for blood flow measurement have been discussed elsewhere (16, 30, 34, 35, 44). The FM method has been validated in numerous organs except bone. When microspheres are used for determination of organ blood flow, various assumptions and limitations of the microsphere technique need to be considered (3, 8, 12, 16, 31).

To minimize stochastic error due to the sphere distribution, 1 × 10⁶ spheres/kg body wt were injected for a single blood flow measurement. These spheres did not alter hemodynamics of the bone. The absence of any difference in blood flow of right and left kidney suggests adequate mixing and uniform distribution of spheres within the blood (4).

Systematic errors during microsphere quantification were minimized for both microsphere techniques, which are routine in our laboratory (21, 32, 43). In contrast to the RM method, we noticed sample loss by measuring fluorescence intensity during automated
sample processing. This was due to the fatty bone marrow, which blocked the SPU filter. Addition of alcohol to the digestion solution prevents this error. Whereas radioactivity can be measured immediately after bone tissue sampling, the FM technique requires decalcification of the bone samples for 3 wk. We previously showed that this procedure does not influence the fluorescent characteristics of the FM (2).

Under the experimental conditions described, we found a highly significant linear correlation between relative blood flow values determined by simultaneous injections of FM and RM in all experiments; the mean difference between the relative bone blood flow estimated by FM and RM was close to zero at 90, 70, and 50 mmHg MAP.

**RBBF.** Previous data from animal studies indicate that bone blood flow is governed by neural, hormonal, and metabolic mechanisms (10, 11, 15, 18, 37, 38). We found equal blood flow values in right and left bone samples, which is consistent with values reported in previous studies (1, 9, 17, 27, 33, 39).

Mean bone blood flow was highest in the humerus (14 ml·min⁻¹·100 g⁻¹), followed by the femur and tibia (11 and 7 ml·min⁻¹·100 g⁻¹, respectively). These data are in contrast to the results of Shim et al. (38), who measured RBBF in long bones of the rabbit by clearance of ⁸⁵Sr. Although they reported no data for hematocrit, we found blood flow values of ~10 ml·min⁻¹·100 g⁻¹ in femur, tibia, and humerus.

Previous studies suggest that regional perfusion within a long bone is heterogeneous (24, 36). About 60–70% of total blood flow in the femur, tibia, and humerus was detected in the metaphyses and diaphyses, which have proportionally higher bone marrow than other regions. Cumming (10) found a mean blood flow of 52 ml·min⁻¹·100 g⁻¹ in bone marrow of the femur, which was measured by venous effluent collection. The discrepancy between this value and values obtained in our study may be explained by the fact that we measured the blood flow in samples containing bone marrow and compact bone. Furthermore, Cumming used young rabbits, whereas we used adult rabbits. It is known that blood flow in marrow is age-dependent: it is lower in older animals (7).

We did not observe a decrease of blood flow in kidneys and bone when MAP was deliberately reduced from 90 to 70 mmHg. Inasmuch as CO remained unchanged at moderate hypotension, this may possibly be explained by a decrease in peripheral vascular resistance in all organs, including bone. This finding suggests that bone vessels have a mechanism to autoregulate flow when arterial pressure is lowered. This might be protective during moderate hypotension. The results of our study are in accordance with those of Michelsen (28), who demonstrated autoregulating responses of bone marrow flow.

The decrease in bone blood flow after reduction of MAP to 50 mmHg indicates the response to acute blood loss (40). We observed a decrease of CO by 30%, whereas the vascular resistance remained unchanged. These findings are similar to those of Gross et al. (18) and Yu et al. (46), who reported a decrease of bone and bone marrow blood flow during hemorrhagic shock. This indicates that perfusion of bones in severe hypotension mainly depends on the perfusion pressure.

The heterogeneity of perfusion within a given bone did not change during hypotension. This observation supports the notion that the different regions of the bone have a common mechanism for control and maintenance of blood flow.

**Conclusion.** We found that the FM reference sample technique was an accurate and reliable method for repetitive measurements of RBBF. Changes in RBBF under deliberate hypotension indicate that vasomotor control mechanisms, as well as perfusion pressure, play a role in controlling RBBF. Application of this method to further investigations may help us better understand the pathophysiology of bone microcirculation.

**DISCLOSURES**

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