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Determination of Regional Bone Blood Flow by Means of Fluorescent Microspheres Using an Automated Sample-Processing Procedure

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Key Words

Bone blood flow · Fluorescent microspheres · Animal experiments

Abstract

The determination of regional blood flow utilizing fluorescent microspheres (FMs) is an established method for numerous organs. Recent progress, in particular the automation of sample processing, has further improved this method. However, the FM method (reference sample technique), which allows repetitive measurement of regional organ blood flow, has so far not been used for the determination of blood flow in bone. The aim of the present study was to establish FM for the quantification of regional bone blood flow (RBBF). Female, anesthetized New Zealand rabbits (n = 6) received left ventricular injections of different amounts of FM at six subsequent time points. In order to examine the precision of RBBF determination, two different FM species were injected simultaneously at the sixth injection. At the end of the experiments the femoral and tibial condyles of each hind limb were removed and the fluorescence intensity in the tissue samples was measured by an automated procedure. In an in vitro study we have shown that acid digestion of the crystalline matrix has no effect on the fluorescence characteristics of FM. The determination of the number of spheres per tissue sample revealed that depending on the tissue sample size up to 3×10^6 spheres/injection were necessary to obtain about 400 microspheres in the individual bone samples. RBBF values of the tibial and femoral condyles did not differ at various injection intervals. The tibial blood flow values varied between 6.6 ± 1.1 and 8.5 ± 1.4 ml/min/100 g and were significantly higher than those of the femur (4.3 ± 1.1 to 6.0 ± 1.8 ml/min/100 g). The bone blood flow values obtained by simultaneous injection of two FM species correlated significantly (r = 0.96, slope = 1.06, intercept = 0.05), the mean difference was 0.39 ± 1.11 ml/min/100 g. Our data demonstrate that the measurement of RBBF by means of FM allows a valid determination of RBBF.

Introduction

In 1967 Rudolph and Heymann [1] introduced radiolabelled microspheres and 1 year later Makowski et al. [2] reported the reference blood sample technique for the determination of regional organ blood flow (RBF). Using this method microspheres are delivered to the different organs by the arterial blood after injection into the left ventricle or atrium. Due to their diameter microspheres are trapped in the capillaries of the organs. During the

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injection of microspheres an arterial reference blood sample is withdrawn by means of a Harvard pump. The concentrations of microspheres in the reference blood sample and in the tissue samples were determined and the blood flow values were calculated.

However, due to the hazards inherent to radiolabelled microspheres, fluorescent microspheres (FMs) [3, 4] and colored microspheres [5, 6] were developed and validated for determination of RBF. These methods require complete recovery of the microspheres from the tissue and reference blood samples. Recent innovations such as the automation of the sample-processing procedure [7] and the development of the sample-processing unit (SPU) [8] have enabled the improvement and standardization of the FM technique for measurement of RBF, which has proven to be the most suitable technique for simultaneous determination of RBF in the heart, lung, kidney, muscle, brain and spleen [3, 4, 9, 10]. Even though the use of FM for assessment of regional bone blood flow (RBBF) [11, 12] has been reported, the suitability of the reference blood sample technique for measurement of RBBF by means of FMs has not been demonstrated so far. In contrast to other organs, the application of the FM technique for bone tissue requires as a first step decalcification of samples with acids which may alter the fluorescence characteristics of the spheres. Therefore an in vitro study was undertaken to evaluate the possible influences of the decalcifying process on the fluorescence characteristics of the spheres.

Errors in blood flow studies using the microsphere reference sample technique are mainly due to stochastic error. To achieve valid blood flow data a sample should contain 384 spheres [13]. The number of spheres lodged in a tissue sample depends on hemodynamic parameters, tissue blood flow, tissue sample size and the number of totally injected spheres. As the sample size and number of spheres injected can be varied easily, we investigated the number of microspheres in large bone samples after injecting increasing dosages of FMs.

Material and Methods

Quantification of the Fluorescence Intensity

Seven differently labelled FM species (FluoSpheres®, Molecular Probes, Eugene, Oreg., USA) were used in this study. FMs with a diameter of 15 μm were delivered in a solution containing 0.15 *M* NaCl and 0.05% Tween 20.

To determine the number of spheres in tissue and reference blood samples, 1 ml of the stock solution (≈10^6 spheres) was diluted with 9 ml of 0.9% NaCl. The number of spheres per microliter of this solution was then determined using a flow cytometer [14]. With this method, impedance alterations dependent on the particle size between electrodes were analyzed and the number of particles was calculated automatically. Measurements were repeated 10 times for each FM species. The solution with a known amount of spheres was used for serial dilutions (7 dilution steps, 1:10 each). The fluorescence intensity of each resulting aliquot was measured and the data used to establish a standard curve. The slope of the standard curve served to calculate the number of FMs in the tissue samples. Each FM species (1 μl of 1 ml from a 1:10 diluted stock solution) was measured 10 times in order to determine the precision and reproducibility of the measurement of fluorescence (LS 50B, Perkin Elmer, Überlingen, Germany).

To test the stability of the FMs in acidic solution we compared the fluorescence intensity of 10-μl aliquots measured directly and after storage for 4 weeks in HCl (1 mol/l). This procedure was repeated 10 times. In order to examine interference from calcium on the automated fluorescence intensity measurement the same procedure was repeated with bone tissue samples.

Surgical Preparation

Six adult, female New Zealand rabbits (Charles River, Kisslegg, Germany) with a mean body weight of 3.4 ± 0.5 kg were used in this study which was authorized by the animal care committee of the local Bavarian government, Munich, Germany. The epiphysial plates were closed in each rabbit, as was verified during dissection of the bones. Preoperatively the animals were kept in groups on the ground and allowed free movement.

Animals were anesthetized by intramuscular injection of ketamine (Ketavet®, 15 mg/kg body weight, Pharmacia & Upjohn, Erlangen, Germany) and xylacin (Rompun®, 2 mg/kg BW, Bayer, Leverkusen, Germany) and allowed to spontaneously breath room air and supplemental oxygen (2 liter/min). Animals were fixed in the supine position, the right common carotid artery was isolated and a catheter (Cavafix® MT, flow rate 10 ml/min; B. Braun Melsungen AG, Melsungen, Germany) inserted. A pressure-monitoring system (Sirecust 304 D, Siemens, Munich, Germany) was connected to the catheter. As this catheter served for the injection of FMs it was advanced into the left ventricle. The correct position of the catheter's tip was identified by the typical waveform of the left ventricular pressure curve. For the collection of the arterial blood sample a second catheter was introduced into the left carotid artery and advanced into the descending aorta. Blood pressure and heart rate were monitored continuously throughout the experiment. Blood losses were compensated by intravenous infusion of 0.9% saline (15 ml/h). Changes in systolic blood pressure of less than ± 15% and in heart rate of less than ± 20 beats/min were tolerated. After the last injection of microspheres the animals were euthanized with an overdose of pentobarbital (Narcoren®, Rhône Mérieux GmbH, Laupheim, Germany).

Measurement of Regional Bone Blood Flow

Prior to each injection, FMs were vortex mixed (Genie 2, Bender and Hobein AG, Zürich, Switzerland) for 3 min and sonicated (Sonorex TK52H, Bandelin, Germany) for 5 min again followed by 3 min of vortex mixing. FM species were selected randomly from the stock solution and diluted 1:10 in 0.9% NaCl.

In order to find the optimum dosage of microspheres needed for a valid measurement, animals were injected as follows: the 1st injection with 0.5 × 10^6 FM; the 2nd with 1.0 × 10^6 FM; the 3rd with 2.0 × 10^6 FM, and the 4th and 5th with 3.0 × 10^6 microspheres. To test

the precision of the measurement, the last injection was carried out with pairs of two FM species (3×10^6 spheres each). Prior to each injection the correct position of the catheter was controlled and blood pressure and heart rate were noted. Each injection lasted for 1 min and was carried out by the same person. A period of 20 min for stabilization was allowed to elapse between two consecutive injections. The withdrawal of the arterial reference blood sample from the catheter in the aorta was started 15 s before the injection of microspheres and continued for 2 min. The withdrawal rate of the Harvard pump ('33' Syringe Pump, FMI, Egelsbach, Germany) was 3.54 ml/min. Processing of reference blood and tissue samples was performed by our robot system [7] using the SPU (Gaiser, Kappel-Grafenhausen, Germany) developed by our group [8].

Sample Processing

At the end of the experiments organs were removed from the animals. To control for adequate mixing of the spheres in the arterial blood, both kidneys were taken and the connective tissue as well as the renal pelvis were removed. Each kidney was then dissected into 8 samples, resulting in a total of 96 samples from all experiments. The average weight of the right and left kidney samples was 1.30 ± 0.37 and 1.30 ± 0.32 g, respectively.

The femoral condyles and tibial plateaus were separated from the rest of the bones. Muscles, periost, ligaments as well as cartilage were removed with a scalpel and a bone curette. Left tibial and femoral bone samples were then divided into medial and lateral condyle. The mean weight of the tibial plateau and femoral condyle was 1.87 ± 0.23 and 2.25 ± 0.27 g, respectively. The mean weight of the medial and lateral tibial condyles was 0.87 ± 0.07 and 0.95 ± 0.16 g, respectively, whereas medial and lateral femoral condyles weighed 1.13 ± 0.26 and 1.05 ± 0.22 g, respectively.

The crystalline matrix of the bone samples was dissolved by soaking the samples in hydrochloric acid (1 mol/l) for a period of 4 weeks. The organic matrix was then digested with a mixture of KOH (4 mol/l), Tween 80 and isopropyl alcohol at 60°C and the FMs isolated by filtering the digested material by means of the SPU according to the protocol of Thein et al. [7]. The tissue sample-processing procedure including sample digestion, FM isolation and online measurement of the fluorescence intensity was carried out by an automated procedure [8].

Samples of kidneys were processed as described above immediately after the dissection. The fluorescence intensity of the arterial reference blood samples was measured after filtering the blood through the filter within the SPU. To render the blood filterable, 1 vol blood was diluted with 2 vol of sodium citrate.

The data from the fluorescence measurements served to calculate the blood flow values (ml/min/100 g) for each tissue sample and injection interval according to the formula:

$$F_{sample} = F_{reference} \times \frac{N_{sample}}{N_{reference}}$$

where: F_{sample} = blood flow in the sample in ml/min; $F_{reference}$ = withdrawal rate of the Harvard pump (3.54 ml/min); N_{sample} = number (intensity) of MS in the sample, and $N_{reference}$ = number (intensity) of MS in the reference sample.

To allow the comparison of different samples, the calculated blood flow values were divided by the tissue weight and normalized to 100 g, giving the blood flow value (ml/min/100 g).

Statistical Analysis

The statistical analyses were carried out with the software SPSS for Windows, Version 11.0 (SPSS Inc., Chicago, Ill., USA). To evaluate the fluorescent intensity per sphere, linear regression analysis was used to estimate the slope summarizing the relationship between the fluorescence intensity and the number of spheres. The coefficient of correlation (r), the coefficient of determination (r^2) and the standard error of the estimate were computed for all pairs. Slopes and intercepts were compared to unity and 0, respectively, for a two-sided 95% confidence interval.

The relative coefficient of variation in percent (rel CV = $CV/n^{0.5} \times 100$) was used to characterize the precision of repeated measurements. To compare the fluorescence intensity in samples treated with and without HCl, Student's test was used. RBBF values determined after simultaneous injection of FMs were compared using least-squares linear regression and the method of Bland and Altman [15].

ANOVA according to Friedman was used to determine the significance of differences in mean RBBF of the repeated injections. The statistical significance of the differences in blood flow values from the femur and tibia and the medial and lateral condyles of the femur and the tibia was assessed using Wilcoxon signed rank test. A p value of <0.05 was considered significant.

Results

Quantification of Fluorescence in Microspheres

The number of spheres per milliliter of the stock solution for various FM species was as follows: $1.07 \pm 0.05 \times 10^6$ for blue; $0.92 \pm 0.03 \times 10^6$ for blue green; $1.25 \pm 0.08 \times 10^6$ for yellow green; $0.32 \pm 0.01 \times 10^6$; $0.56 \pm 0.01 \times 10^6$ for red; $0.38 \pm 0.03 \times 10^6$ for crimson, and $0.81 \pm 0.02 \times 10^6$. The relative CV for counting the microspheres with the flow cytometer ranged between 0.7% (red) and 2.2% (crimson).

To evaluate the fluorescence intensity per sphere linear regression analysis was used to estimate the slope between the fluorescence intensity and the number of spheres in serial dilutions. The within precision values, given as the square of the correlation coefficient, varied between 0.985 and 0.998 (all $p < 0.001$). The confidence intervals of the intercept of all colors included 0. The data of the regression analysis are given in table 1.

Influence of HCl on the Fluorescence Intensity of FMs

In order to examine the possible interference of acid (HCl) which is required to decalcify the bone samples, the fluorescence intensity in FM species was analyzed under defined conditions. The relative CV from 10 repeated measurements of 1 µl of the same aliquot was 0.2% for each FM species. The relative CV calculated from 10 aliquots (10 µl) taken from a diluted stock solution varied between 1.9 and 5.9%. The relative CV for scarlet-colored FM was 2-fold higher than for the other colors. No signifi-

Table 1. Results of the regression analysis between fluorescence intensity and the number of spheres in a serial dilution of each FM species

FM	r	r^2	SEE	Slope (95% CI)	Intercept (95% CI)
Blue	0.998	0.996	10	0.017 (0.015–0.018)	0 (–12–12)
Blue green	0.993	0.985	10	0.016 (0.013–0.018)	11 (–4–25)
Yellow green	0.999	0.998	25	0.052 (0.049–0.055)	26 (–11–63)
Orange	0.996	0.991	7	0.030 (0.026–0.033)	2 (–6–11)
Red	0.999	0.997	4	0.016 (0.015–0.017)	2 (–3–7)
Crimson	0.998	0.995	9	0.044 (0.041–0.048)	–3 (–13–8)
Scarlet	0.998	0.996	16	0.037 (0.034–0.040)	–8 (–27–12)

SEE = Standard error of the estimate.

Table 2. Relative coefficient of variation (in %) of each FM species

Color	FM1	FM1 + HCl	FM2	FM2 + HCl + bone
Blue	3.8	3.8	2.8	2.8
Blue green	2.7	2.8	1.9	1.6
Yellow green	3.1	2.6	2.4	1.4
Orange	3.5	3.4	2.1	1.6
Red	3.4	3.2	2.4	1.6
Crimson	3.5	3.3	3.2	2.6
Scarlet	5.9	5.6	5.5	4.3

Data are calculated by measuring the fluorescence intensity of 10 µl FM1 (n = 10) without HCl and 10 µl FM1 (n = 10) after 4 weeks storage in HCl and from 10 µl FM2 (n = 10) without HCl and 10 µl FM2 (n = 10) after 4 weeks storage in HCl together with bone samples.

Table 3. Number of FMs in the samples of different tissues after injection of 0.5×10^6, 1.0×10^6, 2.0×10^6 and 3.0×10^6, respectively (n = 6; mean ± SD)

Tissues	FMs injected			
	0.5×10^6	1.0×10^6	2.0×10^6	3.0×10^6
Kidney right	$6.4 \pm 2.7 \times 10^3$	$11.3 \pm 4.7 \times 10^3$	$16.0 \pm 6.0 \times 10^3$	$23.2 \pm 10.6 \times 10^3$
Kidney left	$6.4 \pm 2.2 \times 10^3$	$11.8 \pm 3.9 \times 10^3$	$17.1 \pm 4.9 \times 10^3$	$24.0 \pm 11.6 \times 10^3$
FC right	155 ± 46	253 ± 45	399 ± 124	846 ± 288
TC right	213 ± 44	338 ± 73	648 ± 216	1,064 ± 363
FC_{med} left	93 ± 41	140 ± 49	238 ± 104	578 ± 266
FC_{lat} left	54 ± 26	91 ± 46	152 ± 57	376 ± 84
TC_{med} left	109 ± 53	197 ± 69	317 ± 136	609 ± 240
TC_{lat} left	80 ± 52	162 ± 87	291 ± 150	559 ± 324

The number of FMs in the kidneys represents the summation of 8 samples. FC = Femoral condyles; TC = tibial condyles; FC_{med} = medial femoral condyle; FC_{lat} = lateral femoral condyle; TC_{med} = medial tibial condyle; TC_{lat} = lateral tibial condyle.

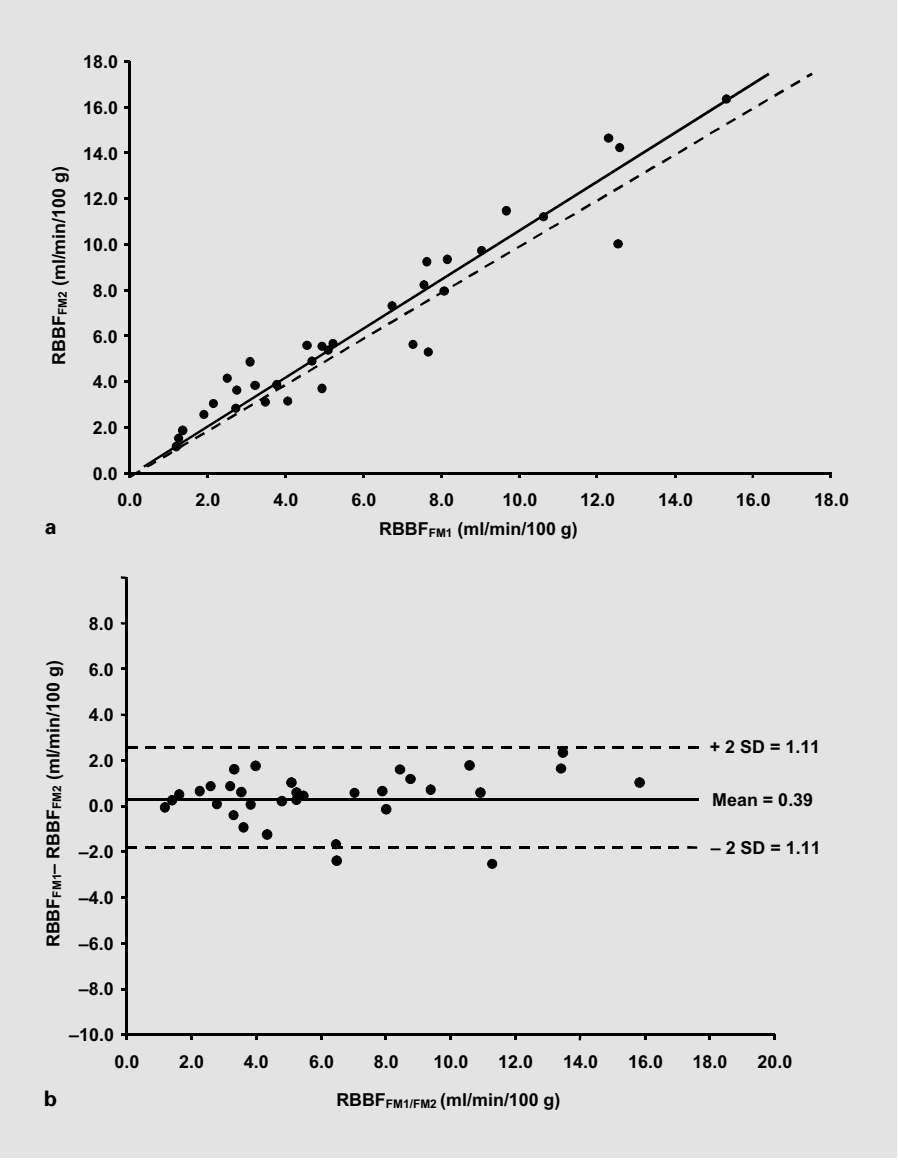

Fig. 1. a Regional bone blood flow values of bone samples obtained by simultaneous injection of two FM species are plotted on x axis and y axis. —— = Slope of linear relationship (n = 35, r = 0.96, r^2 = 0.92, y = 1.06x + 0.05); – – – = isometric line (y = 1x + 0). **b** The Bland and Altman comparison is shown as the difference of the flow determined by FM1 and FM2. —— = Mean difference in ml/min/100 g; – – – = ± 2 standard deviations.

cant differences in fluorescence intensity was noted in samples which were kept in HCl (1 mol/l) with or without bone samples for 4 weeks (table 2).

Number of Spheres in Various Bone Samples

Animals injected with 0.5×10^6 spheres accumulated 213 ± 44 spheres in tibial and 155 ± 46 spheres in femoral condyles. Increasing the number of FMs injected consequently lead to a higher number of microspheres in the bone samples. When 2.0×10^6 FMs were injected 648 ± 216 and 399 ± 124 FMs accumulated in tibial and femoral samples, respectively. Injection of 3.0×10^6 FMs led to accumulation of 609 ± 240 in medial tibial condyle and 559 ± 324 in the lateral tibial condyle, whereas in the medial femoral condyle 578 ± 266 and in the lateral femoral condyle 376 ± 84 spheres were found (table 3).

Reproducibility of Blood Flow Measurements

The blood flow values of the bone samples determined by simultaneous injection of two FM correlated significantly (r = 0.96, r^2 = 0.92, y = 1.06x + 0.05; fig. 1a). The slope was close to 1 and the intercept close to 0. The comparison of Bland and Altman [15] showed a mean difference of 0.39 ± 1.11 ml/min/100 g (fig. 1b).

Regional Blood Flow

For the evaluation of RBBF, data from samples containing 400 spheres are presented in order to ascertain the validity of the measurement [16]. Mean arterial blood pressure ranged between 64 ± 13 (5th injection) and 71 ± 12 mm Hg (2nd injection) but did not change significantly throughout the experiments. Renal blood flow values remained between 2.5 ± 0.9 and 2.9 ± 0.4 ml/min/g. No significant differences were observed between the individual injections and between blood flow values of left and right kidney. Blood flow values of the tibial condyle (6.6 ± 1.1 to 8.5 ± 1.4 ml/min/100 g) and femoral condyle (4.3 ± 1.1 to 6.0 ± 1.1 ml/min/100 g) were not different between the various time intervals during the experiments (fig. 2). The RBBF of the femoral condyle was significantly lower than that of the tibial condyle at each time point (fig. 2). The blood flow of the medial tibial condyle was between 8.0 ± 2.2 and 10.8 ± 2.0 ml/min/100 g and slightly higher than that of the lateral tibial condyle (between 7.5 ± 1.6 and 9.0 ± 2.2 ml/min/100 g). However, these differences were statistically not significant. The medial and lateral femoral condyle blood flow values varied between 5.5 ± 1.5 and 6.8 ± 1.6, and 3.8 ± 1.4 and 5.2 ± 0.3 ml/min/100 g, respectively (fig. 3).

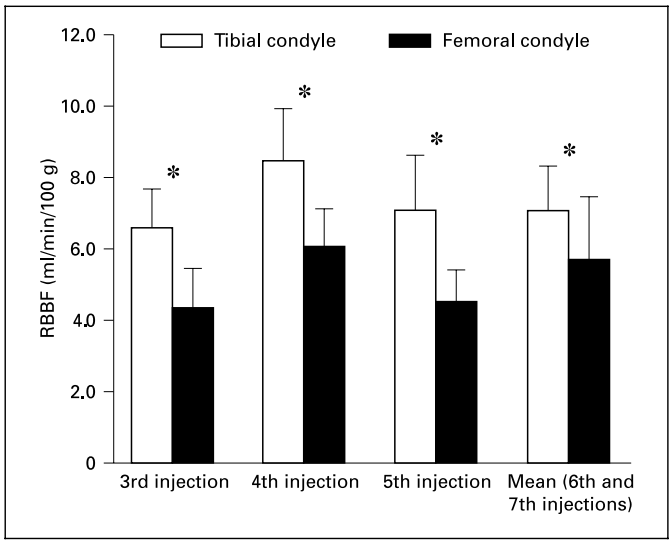

Fig. 2. Blood flow values of the tibial and femoral condyles. Values are given as mean ± SEM. *RBBF of the tibia was significantly higher than in the femur after each injection.

Discussion

Adequacy of the Model

In contrast to other organs, determination of the regional blood flow in the bone is influenced by technical difficulties because of the compact architecture of the tissue. Despite the fact that numerous techniques have been

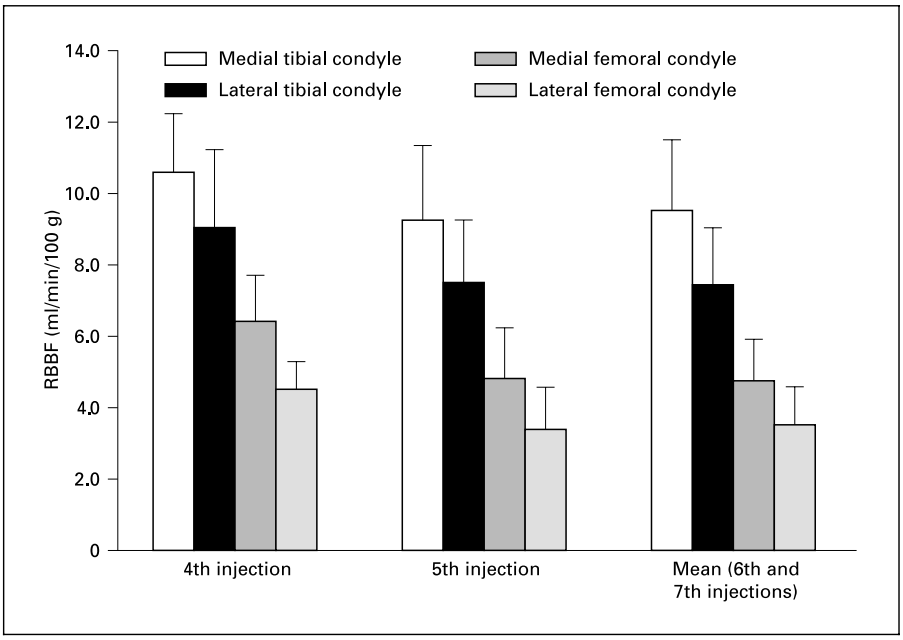

Fig. 3. Blood flow values of the medial and lateral tibial condyles, and the medial and lateral femoral condyles. Values are given as mean ± SEM. Blood flow values of the lateral condyles were slightly lower than of the medial condyles, but the difference was not significant.

applied to study the microcirculation of the bone, only a few have yielded valid RBBF values. The advantages and disadvantages of the different methods have been discussed by several authors [17–20]. The determination of RBBF by microvascular entrapment of microspheres has various advantages. It is possible to carry out repetitive measurements within the same animal without surgical manipulation of the bone. However, to minimize errors in blood flow determination by means of microspheres (reference sample technique), some precautions have to be taken into consideration: (1) microspheres have to be mixed homogeneously in the arterial blood; (2) shunting of microspheres from the site of entrapment should be negligible; (3) arterial blockade of the capillary bed should not alter microcirculation; (4) according to Buckberg et al. [13], 384 microspheres have to be present in an individual tissue sample, and (5) recirculation of microspheres should not occur.

In our experimental setup FMs were injected into the left ventricle to assure homogeneous dispersion of the spheres in the blood [13]. No differences in regional blood flow of the right and left kidneys were observed indicating adequate mixing of FMs in the blood. In contrast to injection of the spheres into the aorta which only allows measurement of the RBBF in the lower extremities [21], injection into the ventricle enables measurement of the RBBF of the thoracic organs and the upper extremities. In addition, the reference blood sample can be obtained via a carotid artery thereby avoiding disturbance of the microcirculation of the hind limb by reference sampling via a femoral artery.

The spheres are completely retained in the capillaries of the lung thereby excluding the possibility of recirculation of spheres [22, 23]. Shunting of spheres has been described in detail [24]. About 1–4% of the injected microspheres with a diameter of 15 μm shunt through the vessels of a healthy bone [25, 26]. We have not observed statistically significant changes in systemic hemodynamic parameters after repeated injections of 15.5×10^6 spheres. This is in agreement with findings of other authors [27, 28].

Errors due to Sample Processing

Although the use of FMs has been validated for numerous organs [3, 4], there are only a few reports on the measurement of bone blood flow [11, 29–31]. In these studies the number of FMs was determined either with a fluorescence microscope [11, 30] or by extracting the fluorescent dyes from the FMs without prior digestion of the tissue [29], which may not assure quantitative recovery of fluorescence from microspheres. In our study the loss of FM was minimized by using the SPU, decalcification and an automated sample-processing technique [7, 8]. The relative CV for measurement of fluorescence was 0.2% for each FM species, indicating that HCl used to dissolve the crystalline matrix did not alter the fluorescence characteristics of the FMs. We observed a higher CV for scarlet-colored FMs. This is in accordance with Schimmel et al. [32], who also found a higher methodological error using scarlet-colored FMs. However, there is still no explanation for this finding.

Number of Spheres per Sample and Precision of RBBF Measurements

In 1971 Buckberg et al. [13] showed that a minimum of 384 microspheres has to be present in an individual sample to achieve reliable blood flow values. However, other authors pointed out that fewer spheres present in an individual sample may still enable the detection of flow heterogeneity [16, 33, 34]. Nevertheless to detect flow changes in single tissue samples as we do, application of the 400 rule is more reliable. Our results indicate that at least 3.0×10^6 FMs have to be injected into rabbits with a body weight of 3 kg to guarantee a sufficient number of spheres per bone sample ($\approx 1.0 \times 10^6$/kg body weight).

To test the precision of our approach, the last FM injection was carried out by injecting two different FM species simultaneously. RBBF values obtained by simultaneously injected FMs showed a highly significant linear correlation. The slope was close to 1 and the intercept was close to 0. The Bland and Altman comparison for all bone samples showed a mean difference of 0.39 ml/min/100 g and a uniform distribution of scatter above and below 0, indicating the validity of the measurement.

The blood flow values of the femoral and the tibial condyles obtained in this study are in accordance with earlier reports [12]. We have confirmed the data of Okubo et al. [35], who also reported higher blood flow values in the tibial as compared to the femoral condyles. In contrast to McGrory et al. [36], we did not observe changes of RBBF over a period of 60 min.

RBBF is influenced by physiological, humoral and neurogenic stimuli. Gross et al. [25], using radioactive spheres, observed a reduction in RBBF under hypotension, hypoxia and acidosis, which they explained by an increase in vascular resistance. The influence of neurogenic factors and the effect of exercise are unclear [25, 37–40]. In order to detect minimal changes in RBBF under physiological and pathological conditions, the experiments should be standardized as much as possible. To

evaluate the systemic hemodynamic conditions at the time of injection, arterial blood pressure and cardiac output should be measured. Cardiac output can easily be determined post hoc by the number of spheres injected, the number of spheres in the reference blood sample and the withdrawal rate of the Harvard pump [23]. However, values of cardiac output calculated in this manner have to be interpreted critically, as the number of spheres in the stock solution may vary after repeated withdrawals of spheres despite adequate vortex mixing and sonication of microspheres. Therefore the number of spheres injected should always be determined by analysing an aliquot of the injected volume.

Conclusions

We could show that decalcification of the bone by means of HCl does not influence the fluorescence characteristics of the FMs. The remaining tissue can be digested completely as reported previously by our group. Injection of at least 2×10^6 FMs is needed to achieve a local deposition of at least 400 spheres in femoral and tibial condyles. For smaller samples, e.g. medial and lateral condyles, injection of 3×10^6 FMs is recommended. These two findings together with complete recovery of FMs from the tissue and reference blood samples as demonstrated here render the FM method a reliable and valid technique for the measurement of regional blood flow in bone tissue.

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