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Assessment of regional bone tissue perfusion in rats using fluorescent microspheres

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ABSTRACT

Disturbances in bone blood flow have been shown to have deleterious effects on bone properties yet there remain many unanswered questions about skeletal perfusion in health and disease, partially due to the complexity of measurement methodologies. The goal of this study was use fluorescent microspheres in rats to assess regional bone perfusion by adapting mouse-specific fluorescent microsphere protocol. Ten fifteen-week old Sprague Dawley rats were injected with fluorescent microspheres either via cardiac injection ($n = 5$) or via tail vein injection ($n = 5$). Femora and tibiae were harvested and processed to determine tissue fluorescence density (TFD) which is proportional to the number of spheres trapped in the tissue capillaries. Right and left total femoral TFD (2.77 ± 0.38 and 2.70 ± 0.24, respectively) and right and left tibial TFD (1.11 ± 0.26 and 1.08 ± 0.34, respectively) displayed bilateral symmetry in flow when assessed in cardiac injected animals. Partitioning of the bone perfusion into three segments along the length of the bone showed the distal femur and proximal tibia received the greatest amount of perfusion within their respective bones. Tail vein injection resulted in unacceptably low TFD levels in the tibia from 4 of the 5 animals. In conclusion this report demonstrates the viability of cardiac injection of fluorescent microspheres to assess bone tissue perfusion in rats.

1. Introduction

Bone blood flow plays a crucial role in bone growth (Fleming et al., 2001), fracture repair (Grundnes and Reikerås, 2009; Maes et al., 2010; Tomlinson and Silva, 2014), and bone homeostasis (Carulli et al., 2013; McCarthy, 2006). Disturbances to bone blood flow have been shown to have deleterious effects on bone health and function (Carulli et al., 2013; Colleran et al., 2000; Prisby et al., 2007; Stabley et al., 2015, 2013) yet there remain many unanswered questions about skeletal perfusion in health and disease, partially due to the complexity of measurement methodologies (McCarthy, 2006).

Radioactive microspheres were long considered the experimental gold standard for the determination of skeletal perfusion (McCarthy, 2006) due to their accuracy and ease of analysis (Anetzberger et al., 2004). Upon injection into the animal, microspheres lodge in tissue capillaries in direct proportion to the fraction of cardiac output perfusing the tissue (McCarthy, 2006). Tissue analysis is relatively straightforward, even for mineralized tissue, in that once tissues of interest are harvested, blood flow is estimated via a gamma spectrometer with minimal tissue processing (Colleran et al., 2000; Kirkeby and Berg-Larsen, 1991; Schimmel et al., 2000; Stabley et al., 2013). Various limitations of radioactive microspheres, including cost, health risks, and the necessity for precautions with use and disposal (Glenny et al., 1993), have resulted in exploration of alternative methods.

Fluorescent microspheres, which work on the same principal as radioactive spheres in that they become entrapped within capillaries, have been shown to allow measurement of organ perfusion as effectively as radioactive microspheres (Glenny et al., 1993). Analysis of fluorescent microspheres requires degradation and filtration of the tissues of interest before the samples can be analyzed and has been effectively used in numerous soft tissue across multiple species (Altemeier et al., 2000; Anetzberger et al., 2004; De Visscher et al., 2003; Glenny et al., 1993; Hlastala et al., 1996). Processing of skeletal tissue presents more challenges for fluorescent microspheres compared to soft tissue, perhaps helping to explain why fluorescent sphere data exists only for mice (Serrat, 2009) and rabbits (Anetzberger et al., 2004).

In the present study, we sought to use fluorescent microspheres in rats to assess regional bone perfusion. The goal was to adapt a fluorescent microsphere protocol used in mice (Serrat, 2009) to determine the variability of skeletal perfusion both within and among a set of

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normal rats, as well as to test various perturbations in experimental methodology.

2. Methods

2.1. Microsphere storage and fluorescence decay

In order to determine characteristics of the microspheres, several perturbations in storage and analyses were carried out prior to animal experiments. The effect of different methods of storage on fluorescence quantification was examined by placing a known amount of microspheres (50,000 beads in 0.05 mL) in either phosphate buffered saline (PBS) or ethanol (EtOH). Additional sets of microspheres were placed in PBS and frozen at −4 °C or −20 °C. Finally, the stability of fluorescence over time after being released from the microspheres was measured over 10 days.

2.2. Animals

Fifteen-week old male Sprague Dawley rats (n = 10) were used for this study. All procedures were approved by the Indiana University School of Medicine Animal Care and Use Committee prior to initiating the study.

2.3. Microsphere injection

Polystyrene, red fluorescent (580/605), 15 μm microspheres (FluoSpheres, ThermoFisher), were used for blood flow determination. Microsphere injection in half of the rats (n = 5) was performed as previously described with minor adaptations for rats (Serrat, 2009). The process is summarized and illustrated in Fig. 1. Briefly, under isoflurane anesthesia, the chest cavity was opened to allow visualization of the heart. A 2.5 mL solution of microspheres (containing one million spheres/mL) was injected in the apex of the left ventricle of the beating heart. The spheres were allowed to circulate for 60 s before the animal was euthanized and tissues collected. The number of microspheres injected was based, on a mg/kg basis, off of using 2×10^5 microspheres for a 43 g mouse which works out to 4.65×10^6 spheres/kg; we rounded up to 5.0×10^6 spheres/kg for ease (Serrat, 2009). A pilot study was done to verify that the number of spheres entrapped in bone vasculature using a 2.5 million sphere injection was within the generated standard curve used to estimate microsphere number. Due to the potential variability of cardiac function after opening the chest cavity, another five rats were administered a similar number of microspheres (2.5 million) via a tail vein catheter. For these animals, microspheres were allowed to circulate for 5 min before the rats were euthanized and tissues collected.

2.4. Sample processing

Femur and tibia samples were divided into proximal, middle (diaphysis), and distal segments as previously described (Colleran et al., 2000). Marrow was left intact in all bone specimens. Bone samples were placed in individual amber vials with 15 mL of Cal-Ex Decalcifier solution. After 4 days, decalcified bone samples were placed in 5% ethanolic potassium hydroxide for degradation. Samples were vortexed every 24 h to ensure complete degradation. After 96 h of degradation, samples were filtered through polyamide mesh filters (5 μm pore size). 1 mL of Cellosolve acetate (2-ethoxyethyl acetate, 98%, Sigma, cat. no. 109967) was added to each of the filtered samples to break the microspheres open and expose the fluorescence.

2.5. Fluorescence quantification

All fluorescence measurements were made using the SpectraMax i3x microplate reader (Molecular Devices, CA). Three 100 μL aliquots from each sample were placed in a 96-well microplate for fluorescence quantification. Red fluorescence was measured using an excitation of 580 nm and an emission of 620 nm. Standard curves of serial dilutions with known amounts of microspheres were generated on the day of analysis. Standard curves generated were used to approximate the number of spheres in a given sample. All data is presented as tissue fluorescence density (TFD) as AU/g and scaled by 10^6.

2.6. Statistical analysis

All analyses were performed using the Statistics Toolbox in MATLAB software. Paired student *t*-tests were utilized to compare right and left TFD measurements of the femora and tibiae. A priori α-levels were set at 0.05 to determine significance. Coefficients of variation (CV) were calculated within each bone segment (using data from both right and left limbs; n = 10).

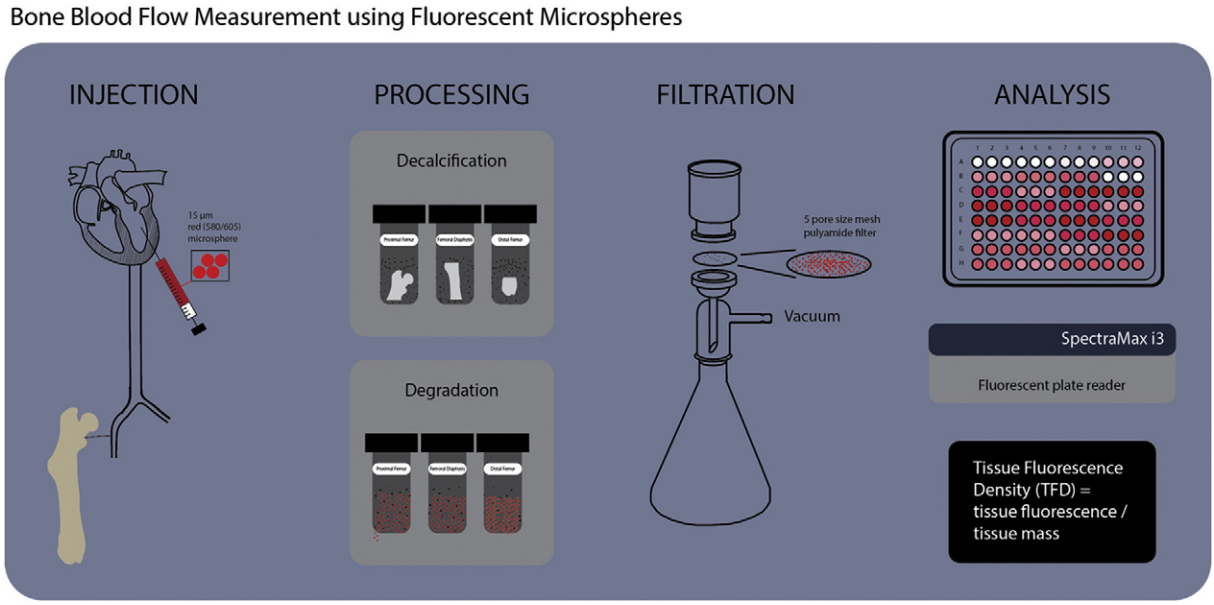

Fig. 1. Graphical depiction of microsphere protocol including microsphere injection, tissue processing and fluorescence quantification.

3. Results

Neither storage of intact microspheres in EtOH or freezing temperatures for 24 h altered fluorescence of the spheres compared to recommended storage in PBS (Fig. 2A). Furthermore, measurement of fluorescence showed no decay after 4 or 10 days relative to measures taken immediately after fluorescence release (Fig. 2B).

Tissue fluorescence density (TFD) data for the various regions of femora and tibia from animals administered microspheres through the left ventricle are presented in Table 1. Right and left total femoral TFD (2.77 ± 0.38 and 2.70 ± 0.24, respectively) from animals with microspheres injected in the left ventricle were not significantly different ($p = 0.75$). Right and left tibial TFD (1.11 ± 0.26 and 1.08 ± 0.34, respectively) were also not different ($p = 0.59$). The coefficient of variation (CV) of TFDs among the five animals was 10.95% and 26.06% for the femur and tibia, respectively. Partitioning of the tissue perfusion into three segments along the length of the bone showed the proximal femur received 27.30 ± 4.04%, the distal femur 51.34 ± 7.97%, and the femoral diaphysis 21.36 ± 6.68% of total femoral perfusion (Fig. 3). The tibia partitioning showed proximal tibia received 68.77 ± 5.86%, the distal tibia 10.68 ± 4.18%, and the tibial diaphysis 20.55 ± 5.86% of total tibial perfusion. Bone segment masses are presented in Supplementary Table 1.

Tail vein injection resulted in unacceptably low levels of tibia perfusion (TFD) in the tibia from 4 of the 5 animals (Table 2). Analyses of femora from these animals were not undertaken.

Table 1
Tissue fluorescence density in the femur and tibia following intra-cardiac injection of microspheres.

Animal	Proximal		Diaphysis		Distal		Total	
	Right	Left	Right	Left	Right	Left	Right	Left
Femur								
1	2.01	2.17	1.08	1.27	5.02	5.57	2.73	2.80
2	1.78	1.86	2.40	1.40	4.19	4.37	2.77	2.63
3	2.30	2.87	1.64	1.72	4.15	3.75	2.84	2.90
4	3.10	2.50	3.24	2.45	3.51	3.51	3.28	2.83
5	1.73	1.61	1.84	1.78	3.13	3.60	2.22	2.31
Mean	2.18	2.20	2.04	1.72	4.00	4.16	2.77	2.70
Stdev	0.56	0.50	0.82	0.46	0.72	0.86	0.38	0.24
CV	0.23		0.34		0.18		0.11	
Tibia								
1	2.45	2.51	0.63	0.39	1.40	1.06	1.51	1.37
2	2.25	2.49	0.49	0.59	0.57	0.81	1.13	1.44
3	1.14	1.46	0.60	0.49	0.52	0.46	0.81	0.87
4	2.04	1.14	0.55	0.23	0.52	0.26	1.12	0.63
5	1.45	1.75	0.60	0.71	0.57	0.39	0.96	1.10
Mean	1.87	1.87	0.57	0.48	0.72	0.60	1.11	1.08
Stdev	0.55	0.61	0.06	0.19	0.38	0.33	0.26	0.34
CV	0.29		0.26		0.52		0.26	

Tissue fluorescence density (TFD) data presented as AU/g and scaled by 10^6. CV = coefficient of variation among right and left (combined together so n = 10 specimens per CV) bone segments across all five animals.

4. Discussion

The importance of bone blood flow in skeletal health, and its role in disease has been established through several preclinical experiments. Although, the gold standard for bone blood flow measurement has been the injection of radioactive microspheres, fluorescent microspheres have been established as a safer and equally effective technique for non-skeletal tissues (such as lung, kidney, and brain). Measures of bone blood flow using fluorescent microspheres presents challenges due to the difficulty of imaging through mineralized tissue and the technical difficulty of current microsphere assays, especially in small animals. The current work extends the use of fluorescent microspheres, described in detail for mice (Serrat, 2009), to assess bone perfusion in rats.

Although the use of fluorescent microspheres has been well detailed in mice (Serrat, 2009), some protocol adaptations were needed to measure fluorescent microspheres in rat. A larger absolute number of microspheres was used based on the recommendation of approximately 5 million spheres/kg of body weight used successfully in mice (Serrat, 2009). Due to larger volume of tissue in rat compared to mouse, larger volumes of ethanolic KOH were needed to degrade the tissue. Segments of bone were placed in 15 mL of 10% ethanolic KOH (vs 8 mL of 3% ethanolic KOH in mice). Also due to the larger tissue volume, a larger vacuum filtration apparatus was necessary to filter the degraded samples. Degraded samples were filtered through 47 mm polyamide filters (versus 25 mm filters used in mice).

Our results on tibia and femoral perfusion match closely with previous reports examining values across these two bones as well as regionally within each bone. The ratio of femoral to tibial perfusion reported in the current work matches the results of previous studies performed using microspheres in rats (MacPherson and Tothill, 2012; Kapitola and Zák, 1998) and dogs (Okubo et al., 1979). The percentage of tissue perfusion to the different segments of the femur and tibia reported in our work also match values using radioactive microspheres showing highest flow in distal femur and proximal tibia (Schoutens et al., 1979). This congruence with previous work lends support to the

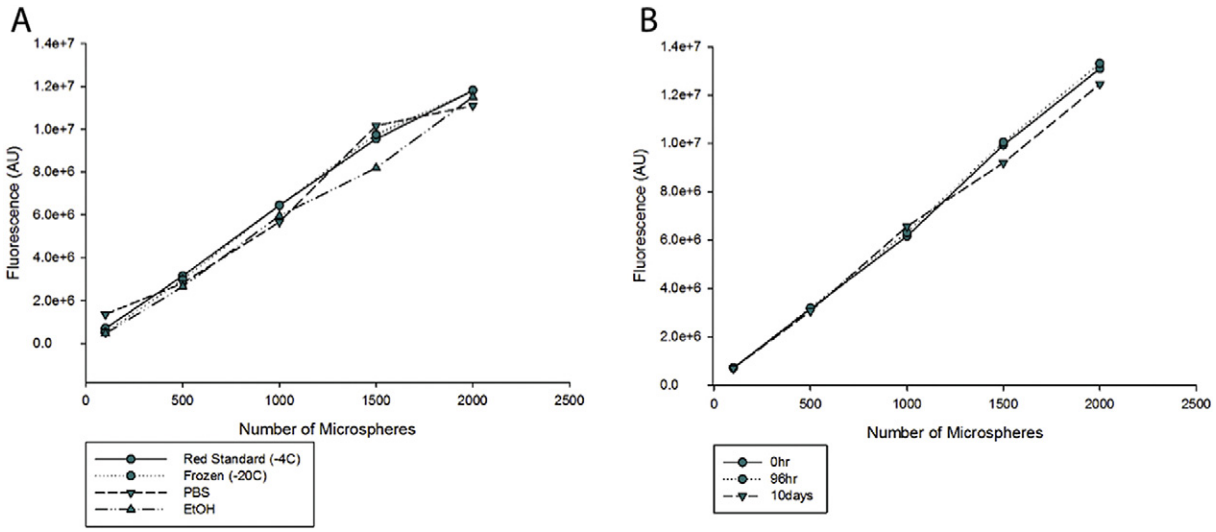

Fig. 2. Microsphere standard curves displayed as bead quantity and fluorescence. (A) Effects of storage conditions. (B) Effects of time.

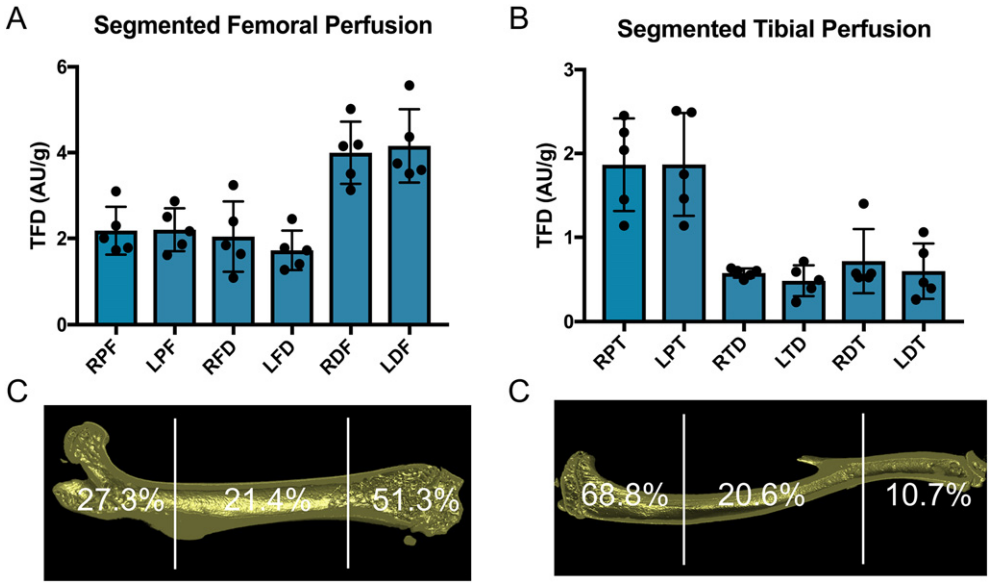

Fig. 3. Tissue fluorescent density (TFD) of segmented femur (A, C) and tibia (B, D). RPF, right proximal femur; LPF, left proximal femur; RFD, right femoral diaphysis; LFD, left femoral diaphysis; RDF, right distal femur; LDF, left distal femur; RPT, right proximal tibia; LPT, left proximal tibia; RTD, right tibial diaphysis; LTD, left tibial diaphysis; RDT, right distal tibia; LDT, left distal tibia. Data presented as means and standard deviations.

viability of the fluorescent microsphere technique for assessing bone perfusion in rats.

Many regional organ perfusion protocols using microspheres require technically challenging surgical techniques including catheter placement into the left ventricle to inject microspheres, and another catheter placement in the carotid artery or the tail artery to collect a reference blood sample. These techniques are further complicated when performed in rodents, given the smaller size of vessels. Because of potential variability in several steps of the sphere administration process, it is worthwhile to find an alternative way to assure adequate spheres are administered. The use of blood as a reference tissue has been used extensively, but other protocols have used organs as the reference tissue (Serrat, 2009). The advantage of using an organ as a reference tissue is it eliminates the need for catheter placement in vessels while the animal is alive. While this will work for interventions that are isolated (such as limb cooling used in the mouse protocol (Serrat, 2009)), our end-goal of this work is to study skeletal perfusion in the context of systemic disease which will likely affect blood flow systemically (and potentially differently across skeletal sites).

In the current study, microspheres were injection into the left ventricle after opening the chest cavity while the animal was under anesthesia as a terminal procedure. Variability in cardiac function before the animal is euthanized due to invasion of the chest cavity was not assessed in the present study and could account for variability among animals. In an attempt to reduce this variability, injections were performed in the tail vein for a subset of animals in order to allow the animal to maintain normal cardiac function to distribute the spheres systemically. Although it was assumed that spheres would be entrapped in the pulmonary capillaries, if enough microspheres passed through to the arterial system, bone perfusion could be measured while also preserving cardiac function. Our results clearly showed that microsphere entrapment in bone was insufficient for reliable measure and thus this is not a viable method of administration.

Table 2
Tibia tissue fluorescence density following cardiac versus tail vein microsphere injection.

Animal	Cardiac injection		Animal	Tail injection	
	Right tibia	Left tibia		Right tibia	Left tibia
1	1.51	1.37	6	1.04	1.34
2	1.13	1.44	7	0.04	0.02
3	0.81	0.87	8	0.03	0.01
4	1.12	0.63	9	0.02	0.01
5	0.96	1.10	10	0.03	0.02
Mean	1.11	1.08		0.23	0.28
Stdev	0.26	0.34		0.45	0.59

Tissue fluorescence density (TFD) data presented as AU/g and scaled by 10^6.

In the present study, we have shown that the injection of fluorescent microspheres in the left ventricle of rats is an effective method for the estimation of bone perfusion. However, there are a number of limitations to this study. Due to the fact that we did not collect a blood sample during injection, we are unable to calculate an estimated flow rate to bone. Although a flow rate provides an easily interpretable value (mL/min per tissue weight), it is worth noting that collection of reference blood from the tail artery, as many do (McDonald et al., 1992; Bloomfield et al., 2002), likely underestimates the concentration of spheres in organs since it is distal to a majority of the arterial tree including highly vascularized tissue including the brain and kidneys. Another limitation to our study is the lack of hemodynamic stability during the microsphere injection procedure and microsphere circulation. The animals are anesthetized using isoflurane, which is known to affect organ perfusion (Bernard et al., 1991) and cardiovascular dynamics (Bernard et al., 1990). Finally, without the use of assisted ventilation, this is a terminal procedure and does not allow for the serial injection of microspheres for the evaluation of longitudinal change in skeletal perfusion. This could be overcome by administration of spheres via prolonged or repeated catheterization if longitudinal measures are of interest.

Although the use of fluorescent microspheres is emerging as an accepted alternative to radioactive microspheres as a method for the measurement of blood flow, and some papers have used the technique to examine bone, there has yet to be a study to assess these techniques in rats. The current work fills this void by showing the viability of injection and analysis of fluorescent microspheres to assess regional bone tissue perfusion in rats.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bonr.2017.04.004.

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