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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).

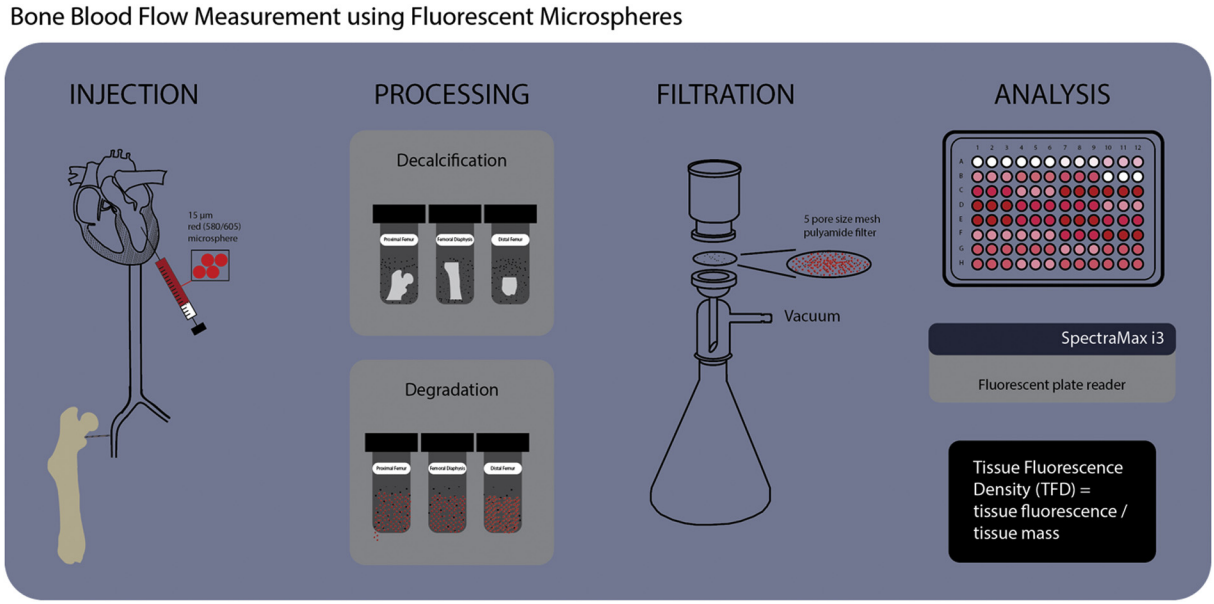

Fig. 1. Graphical depiction of microsphere protocol including microsphere injection, tissue processing and fluorescence quantification.