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Validation of Histologic Bone Analysis Following Microfil Vessel Perfusion

D Sarhaddi, B Poushanchi, M Merati, C Tchanque-Fossuo, A Donneys, J Baker, and SR Buchman

The University of Michigan: Craniofacial Anomalies Program, Pediatric Plastic Surgery, 1500 E. Medical Center Drive, F7894 Mott Children's Hospital, Ann Arbor, Mich. 48109-5219

Abstract

The ability to examine bone vascularity using Micro-Computed Tomography (μ CT) following vessel perfusion with Microfil[®] and to subsequently perform histologic bone analysis in the same specimen would provide an efficient method by which the vascular and cellular environment of bone can be examined simultaneously. The purpose of this report is to determine if the administration of Microfil[®] precludes accurate histologic assessment of bone quality via osteocyte count and empty lacunae count. Sprague-Dawley rats ($n=6$) underwent perfusion with Microfil[®]. Left hemi-mandibles were harvested, decalcified and underwent vascular analysis via μ CT prior to sectioning and staining with Gomori's Trichrome. Quantitative Histomorphometric evaluation was performed. Ninety-five percent confidence intervals were used to determine statistical differences from an established set of controls ($n=12$). Histologic analyses were successfully performed on specimens that had undergone previous perfusion. Quantitative measures of bone cellularity of perfused versus control specimens revealed no statistical difference in osteocyte count per high-power field (95.33 versus 94.66; 95 percent CI, -7.64 to 6.30) or empty lacunae per high-power field (2.73 versus 1.89, 95 percent CI, -1.81 to 0.13). Here we report a statistical validation allowing for histological analysis of cell counts in specimens in which Microfil[®] perfusion has previously been performed.

Keywords

Cellularity; Histology; Mandible; Micro-Computed Tomography; Microfil; Perfusion; Vascularity

Introduction

The process of bony healing involves extensive integration of vessel and bone formation.¹ As a result, studies of bony healing necessarily require examination of the vascular architecture and cellular environment of normal bone in order to establish comparisons and provide comprehensive assessments. As angiogenesis is intricately associated with osteocyte

Corresponding Author: Deniz Sarhaddi, phone: 202.294.1553, denizs@med.umich.edu, fax: 734.936.7815.

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development and survival, many investigations of bone may benefit from the ability to look into these two measures simultaneously.

Currently, accepted techniques for assessing tissue vascularity in small animal models have proven to be challenging as they are time-intensive and yield only limited information. Although descriptive measures of vascular networks are provided by microsphere injection followed by scintigraphy,² the method is limited, as it is primarily designed to measure flow in larger animal models. A more appropriate technique for evaluating bone vascularity for small animal models is histologic staining for endothelial cell markers followed by serial slide quantification.³ This method, however, is extremely time-intensive, is only two dimensional, and provides only an indirect quantitative outcome of vasculature. Importantly, the destructive nature of histology precludes any further three-dimensional study of those specimens.

The utility of Microfil[®], a lead-based silicone elastomer, for the study of vascular networks is well-documented.⁴⁻⁹ Particles of Microfil[®], measuring at 1 μ m, fill the microvasculature and cure to form a 3-dimensional, radio-opaque cast. Our lab has previously demonstrated the utility of using Microfil[®] in conjunction with micro-computed tomography (μ CT) in the murine mandible to accurately and efficiently measure vessel volume, projection, and stereology.¹⁰⁻¹² The combination of Microfil[®] perfusion with μ CT analysis provides the benefit of circumventing the difficulties and shortcomings associated with previous methods by providing a highly accurate, non-destructive and efficient assessment of otherwise difficult to study vascular networks in bone, while leaving specimens intact for further evaluation. Importantly, Duvall and colleagues demonstrated that vascular perfusion with Microfil[®] followed by radiomorphometric analysis using μ CT is not only more accurate and efficient than using immunohistochemistry with serial slide quantification, but it also provides the investigator with a far more robust data set and more control in defining the exact volume of interest (VOI) to be analyzed.¹³

Vascular examination of bone is a valuable additional measure to histologic tissue investigation for *in vivo* studies given the tight coupling of the processes of angiogenesis and bone formation. However, few studies have specifically examined the possible effect that the presence of a lead-based agent, such as Microfil[®], would have upon subsequent histologic studies of bone.

Histologic studies of previously perfused specimens can provide a direct correlation between the cellular environment and vascular microarchitecture within the same specimen. This combination of methods provides an additional level of control for inter-animal variations, allowing for true statistical comparison, while decreasing the number of animals required for any given study. Yamada and colleagues examined this novel method for analyzing angiogenesis within newly augmented bone.¹⁴ We examined this combination of outcomes in a control murine mandibular model with and without Microfil[®] perfusion prior to histologic sectioning and analysis to investigate and validate this methodology. The purpose of this report is to determine if the administration of Microfil[®] precludes the accurate histologic assessment of bone quality via osteocyte count and empty lacunae count.

Materials and Methods

Pre-Operative Animal Care

Male Sprague-Dawley rats ($n=6$), each weighing approximately 400 g., were obtained via the University of Michigan's University Lab Animal Medicine (ULAM) department in compliance with the University of Michigan's Committee for the Utilization and Care of Animals (UCUCA).

Microfil® Perfusion

Experimental animals were anesthetized via inhalation of a 3:1 isoflurane/oxygen mixture. After induction of anesthesia, animals underwent thoracotomy with catheterization of the left ventricle, and ligation of the descending aorta. Vasculature was flushed completely with heparinized normal saline (1000 U/ml) at a pressure of approximately 150mmHg. Specimens were then pressure fixed with formalin, ensuring euthanasia. This was followed by perfusion of Microfil® MV-122 (Flow Tech, Carver, Mass.): a yellow, radiopaque, lead-based, silicone rubber contrast agent. Adequate perfusion was determined and verified by gross examination of the tongue and eyes, for color change to that of Microfil®, indicating complete perfusion of those distal capillary networks. Carcasses were kept at 4°C for 24 hours to allow for complete solidification of Microfil®.

Harvest, Decalcification and Imaging of Hemi-Mandibles

Left hemi-mandibles were harvested, cleaned completely of soft tissues, and stored in 70% ethanol at 4°C until further use. Decalcification of hemi-mandibles was carried out by storing mandibles separately in Cal-Ex®II solution (Fisher-Scientific, Fair Lawn, NJ) with gentle continuous agitation for approximately 4-7 days. Confirmation of demineralization was carried out radiographically with serial daily Faxitron X-Ray scans. After decalcification, μ CT was used to image mandibles to generate quantitative metrics of vascularity as previously described (Figure 1).¹⁰⁻¹²

Histologic Processing

After μ CT analysis, the left hemi-mandibles were vacuum processed via dehydration through graded alcohols and xylene cleaning. Paraffin infiltration was carried out using Paraplast Plus® embedding medium (McCormick Scientific, Richmond, IL) under a 48h program in the Hypercenter® tissue processor (Shandon, Hypercenter XP, Pittsburgh, PA). The specimens were then infiltrated for 2 hours in a vacuum bath (Leica Embedding Center, Model EG1160, Germany), followed by embedding in Paraplast Plus® within 22×22mm Peel-A-Way® embedding molds (Polysciences Inc., Washington, PA). Embedded specimens were stored overnight at 4°C. Blocks were sectioned coronally, from anterior to posterior, into 7 μ m thick serial-sections taken on a Leica Reichert-Jung microtome (Model 2030 Biocut, Germany) using a steel microtome knife (C.L. Sturkey, Inc., Perkiomenville, PA), which provides adequate support for sectioning bone. If difficulty in sectioning perfused specimens was encountered, the specimens were iced to make the block, tissue, and Microfil® easier to cut-- this method was chosen over surface decalcification to avoid altering the tissue. Sections were then mounted on glass slides (Fisherbrand Superfrost®).

Plus, Fisher Scientific, Pittsburg, PA), and were surface stained with Gomori's one-step trichrome.¹⁵

Histomorphometric Evaluation

Point counting of osteocytes and empty lacunae was performed using a light microscope interfaced with a digital camera connected to a computer (Figure 2). Our VOI was superimposed on the digital image using the imaging analysis software program Bioquant[®] NOVA Osteo version 7 (R&M Biometrics, Nashville, TN). Nine high-power field (HPF) images were randomly selected per VOI using 16× magnification. The HPF images measure 640×480 and were stored as TIFF files. Manual point counting of osteocytes and empty lacunae was performed by three reviewers independently.

All data was compared to a previously established non-perfused control group (n=12), which was maintained, prepared and analyzed in exactly the same manner as the perfused specimens.

Statistical Analysis

Confidence intervals (95%) and Independent samples t-test were used to determine statistical differences from a previously established set of controls (n=12) as follows: osteocyte count per HPF and empty lacunae count per HPF. Results were accepted as statistically significant at a value of $P < 0.05$.

Results

No statistical differences were found between Microfil[®] perfused mandibles and nonperfused specimens for osteocyte count per HPF (95.33 ± 8.73 vs. 94.66 ± 5.32 , $P = .841$), 95% CI [-7.64 to 6.30] or empty lacunae per HPF (2.73 ± 1.30 vs. $1.89 \pm .68$, $P = .087$), 95% CI [-1.81 to 0.13] (Figure 3).

Discussion

The quantification of vascularity and angiogenesis is a vital outcome measure for studies across numerous disciplines, particularly when bolstered with coinciding histologic findings. Perfusing specimens with Microfil[®] for μ CT studies of vascularity, and subsequently using the same specimens for histologic analysis is a relatively new technique. This technique is attractive to investigators as it provides a highly efficient, objective and accurate means by which stereologic, quantitative studies of vascularity can be performed, followed by histologic analysis within the same specimens. Laboratories have taken advantage of this efficient and economic combination of outcomes to examine a variety of different tissues ranging from tissue engineered⁵⁻⁶ and grafted tissue⁷⁻⁸, to liver⁹ and bone^{4,10-12}. However, there is a clear paucity of information in the literature regarding the accuracy and statistical validity of this combination of techniques. With this study, we established the validity of performing histology on bony specimens that were previously perfused with Microfil[®].

Conclusion

We found that specimen perfusion with Microfil® allows for vascular study by means of μ CT while allowing for subsequent, undisturbed assessment of bone cellularity via histology.

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Figure 1. Microfil[®] perfused mandibular specimen

Gross image of a decalcified, translucent mandibular specimen (top)- the yellow Microfil[®] is grossly appreciable, highlighting the vascular network. Below is a Microview[®] generated Maximum Intensity Projection (MIP) of the intricate micro-vascular network of the perfused mandible. Despite sufficient decalcification of the mandible, the incisor (In) often retains some degree of radio-opacity, evident in the MIP image.

Figure 2. 16× magnification of Gomori Trichrome stained section of murine mandible
There are no appreciable differences in staining quality between the unperfused sample (top) and Microfil[®] perfused sample (bottom). The perfused sample exhibits the presence of Microfil[®] within the lumen of several blood vessels (M). Marrow Cavity (C), Osteocytes (O), Empty Lacunae (E), Blood Vessel (B).

Figure 3. Osteocyte and Empty Lacunae Counts

No significant difference was found in osteocyte count between control and perfused mandibles (95.33 ± 8.73 vs. 94.66 ± 5.32 , $P = .841$) or in empty lacunae count between the two groups (2.73 ± 1.30 vs. $1.89 \pm .68$, $P = .087$), thereby validating the combination of Microfil perfusion and subsequent histologic studies of bone cellularity.