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A method for isolating high quality RNA from mouse cortical and cancellous bone

Natalie H. Kelly, M.S.^{a,b}, John C. Schimenti, Ph.D.^c, F. Patrick Ross, Ph.D.^d, and Marjolein C.H. van der Meulen, Ph.D.^{a,b,c}

Natalie H. Kelly: nhk28@cornell.edu; John C. Schimenti: jcs92@cornell.edu; F. Patrick Ross: rossf@hss.edu; Marjolein C.H. van der Meulen: mcv3@cornell.edu

^aSibley School of Mechanical and Aerospace Engineering, Cornell University, 105 Upson Hall Ithaca, NY 14853, USA

^bDepartment of Biomedical Engineering, Cornell University, 101 Weill Hall Ithaca, NY 14853, USA

^cCollege of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA

^dResearch Division, Hospital for Special Surgery, 541 East 71st St. New York, NY 10021, USA

Abstract

The high incidence of fragility fractures in cortico-cancellous bone locations, plus the fact that individual skeletal sites exhibit different responsiveness to load and disease, emphasizes the need to document separately gene expression in cortical and cancellous bone. A further confounding factor is marrow contamination since its high cellularity may effect gene expression measurements. We isolated RNA from cortical and cancellous bone of intact mouse tibiae, and also after marrow removal by flushing or centrifugation. RNA isolated from cancellous bone by each method was sufficient for gene expression analysis. Centrifugation removed contaminating cells more efficiently than flushing, as indexed by histology and decreased expression of *Icam4*, a highly expressed erythroid gene. In contrast, centrifuged cortical bone had 12- and 13-fold higher expression of the bone-related genes *Colla1* and *Bglap*, while levels in marrow-free cancellous bone were 30- and 31-fold higher when compared to bone where marrow was left intact. Furthermore, cortical bone had higher expression of *Colla1* and *Bglap* than cancellous bone. Thus, RNA isolated by this novel approach can reveal site-specific changes in gene expression in cortical and cancellous bone sites.

Keywords

osteoporosis; animal models; gene expression; cancellous bone; cortical bone

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Corresponding author: Marjolein C. H. van der Meulen, Ph.D., Sibley School of Mechanical and Aerospace Engineering, Cornell University, 219 Upson Hall, Ithaca, NY 14853, Tel: (607)-255-1445, Fax: (607)-255-1222, mcv3@cornell.edu.

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DISCLOSURES

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1. INTRODUCTION

Osteoporosis is a skeletal disease characterized by low bone mass that often results in fracture. A majority of osteoporotic fractures occur at cortico-cancellous sites such as the proximal femur, distal forearm, and vertebra [1, 2]. At these sites bone is a composite tissue consisting of an outer cortical shell surrounding an inner cancellous network. Mouse models have been developed to understand the etiology of osteoporosis and to identify possible treatments for osteoporosis-related bone decline. Biophysical stimuli, including mechanical loading, are a promising approach to maintain or recover bone mass. In vivo loading often elicits a greater response in cancellous than cortical bone sites, increasing metaphyseal cancellous bone volume fraction by 49–73% and diaphyseal cortical area by 41% in the mouse tibia [3, 4]. But different loading protocols can alter cancellous and cortical bone responses selectively; some stimulate cancellous bone to a greater extent, while others mainly affect cortical bone [5], indicating that differences in mechanoresponsiveness may exist in cortical and cancellous bone. To identify the nature and degree of change in these two bone tissues requires definitive data on which genes are altered at cortical and cancellous sites following manipulation of test animals. To date this information is largely unknown, as most studies have used approaches that are derived from homogenized bone tissues.

The cellular and molecular mechanisms driving load-induced anabolism at the tissue level have been examined using both quantitative real-time polymerase chain reaction (qPCR) and microarray [6–9]. These studies have examined tissues from whole bones, homogenizing cortical and cancellous bone. In contrast, only a single study examined the loading response in cancellous bone from the mouse vertebrae, but did not compare the response to cortical bone [10]. Furthermore, marrow cells have been left in place [8], removed by flushing [7], centrifugation [6, 9], or sequential collagenase digestion [10, 11]. Collagenase digestion has been shown to affect expression of bone-related genes [12], but this effect may depend on the type of collagenase used; endotoxin-free collagenase had a less detrimental effect on RNA quality [13]. However, failure to remove the highly cellular marrow component may result in extensive contamination of RNA isolated from unfractionated bone. The relative efficacy of flushing to centrifugation has not been studied, nor has the degree of bone marrow presence on gene expression. More importantly, gene expression has not been reported in minimally contaminated cortical versus cancellous bone, a challenging endeavor in murine models.

We describe a method to isolate high quality RNA from cortical and, separately, cancellous bone with almost no contamination from marrow cells. Both the quantity and quality of the RNA are suitable for transcriptome studies, providing the ability to compare cancellous and cortical tissues to elucidate differences in cellular and molecular mechanisms at these two sites.

2. MATERIALS AND METHODS

2.1 Animals

Fifteen 11-week-old male C57Bl/6 mice were euthanized and their tibiae rapidly dissected for histology (left leg) or RNA isolation and analysis (right leg). The epiphysis, all soft tissues, periosteum, fibula, and the very distal end were removed. In five mice, bone marrow of the tibiae was not removed (no marrow removal, NMR group). In another five mice, marrow was flushed with 1mL of phosphate buffered saline (PBS) by inserting a 26-gauge needle into the distal tibia. The tibiae in the third group were centrifuged for 20s at 16,100g at room temperature in nested microcentrifuge tubes, where the inner tube (0.2mL) had holes created with a 26-gauge needle to allow marrow to travel into the outer tube (0.65mL). Tibiae were cut approximately 5 mm distal to the growth plate to isolate the metaphysis. The metaphysis was held with forceps so the proximal end was perpendicular to a 1mm biopsy punch (Miltex, Integra LifeSciences Corp, Plainsboro, NJ, USA), which was used to mechanically separate a cancellous core from the cortical shell (Figure 1). The IACUC of Cornell University approved all animal procedures.

2.2 Histology

Cancellous and cortical samples were placed in 10% formalin for 24 hours for fixation, and decalcified in 45% formic acid for 4 days with daily exchange of the solution. Samples were processed and embedded in paraffin and sectioned longitudinally (6µm sections, Leica RM2255, Buffalo Grove, IL, USA). Sectioned samples were stained with hematoxylin and eosin (H&E).

2.3 RNA isolation from bone

RNA isolation was performed using Trizol (Life Technologies, Carlsbad, CA, USA) and RNeasy Mini kit (Qiagen, Germantown, MD, USA) as described previously [8]. Briefly, cancellous and cortical samples (n=30) were pulverized in liquid nitrogen-cooled flasks (Mikro-dismembrator S, Sartorius Stedim Biotech, Bohemia, NY, USA). Following pulverization, Trizol (Life Technologies) was added to the flasks and the powdered bone/Trizol mix was incubated at room temperature for 45 minutes. 300µL of chloroform was added to samples, vortexed for 15s, and decanted into phase lock gel tubes (PLG, heavy, 5 Prime, Gaithersburg, MD, USA). Samples were centrifuged for 15 minutes at 4C and 11,500 rpm, to separate the nucleic acid phase (~600µL), which was removed and added to an equal volume of 70% ethanol. This mixture was applied to purification columns (RNeasy Mini kit, Qiagen) following the manufacturer's instructions, including a DNase digestion (RNase free DNase kit, Qiagen). A final volume of 30µL of RNA was eluted, and purity and quantity were tested using a spectrophotometer (Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA) and RNA Quality Number (RQN) using a fragment analyzer (Advanced Analytical Technologies, Inc, Ames, IA, USA).

2.4 RNA isolation from marrow

Marrow from centrifuged bones (n=3) was placed into RNAlater (Qiagen) at 4C until RNA isolation. Samples were centrifuged for 5 minutes, RNAlater was removed, and 600µL of

lysis buffer with β -mercaptoethanol (Buffer RLT, Qiagen) was added (as described in RNeasy Mini instructions). Samples were homogenized by passing through an 18-gauge needle, centrifuged, and the supernatant (~600 μ L) was added to ethanol and applied to columns for RNA purification as described above.

2.5 Gene Expression

RNA was reverse transcribed to cDNA following the manufacturer's instructions (High Capacity cDNA Reverse Transcription kit, Life Technologies) and brought to 5ng/ μ L with RNase-free water. A final volume of 20 μ L containing 2X SYBR Green (Perfecta SYBR Green Fastmix, with ROX, Quanta Biosciences, Gaithersburg, MD) was assayed by triplicate qPCR using 40 cycles of denaturing (95C, 15s) and annealing/elongation (60C, 60s) (7300 Real-Time PCR System, Life Technologies). Primers for genes that are highly expressed in bone (Collagen type 1 alpha 1, *Col1a1*, and bone gamma-carboxyglutamic acid-containing protein, *Bglap*) and marrow (intracellular adhesion molecule 4, *Icam4*), were designed (available on request). Cycles to threshold (C_T) was determined for each gene and compared to the reference gene *Gapdh*. NMR, flushed and centrifuged groups and marrow samples are presented as relative expression compared to *Gapdh* (2^{-C_T}). Fold-change to cortical and cancellous NMR groups was calculated by the 2^{-C_T} method [14].

2.7 Statistics

To test differences in relative gene expression between cancellous and cortical bone sites, and fold-change compared to NMR, a two-factor ANOVA (main effects: marrow removal and site) with interaction (marrow removal*site) was used with Tukey's HSD post-hoc (JMP Pro 10, SAS, Cary, NC, USA). To test for differences in gene expression between marrow and cancellous and cortical bone with marrow, a one-way ANOVA was used with Tukey's HSD post-hoc. Significance was set at $p < 0.05$.

3. RESULTS

3.1 Histology

To determine if centrifugation was more effective than flushing, we performed histology with H&E of longitudinal sections of cancellous and cortical bone (Figure 2). Cortical (top panels) and cancellous bone (bottom panels), contained significant marrow that was removed incompletely by flushing. In marked contrast, marrow was barely detectable in centrifuged samples.

3.2 RNA Quantity/Quality

We also determined if sufficient, high quality RNA could be extracted from cortical and cancellous bone separately and the effect of marrow contamination on RNA quantity. Intact bone had more RNA than flushed or centrifuged bone. The mean ($\pm$ SD) concentration of cortical samples was 447 ($\pm$ 70), 279 ($\pm$ 13) and 45 ($\pm$ 37) ng/ μ L for NMR, flushed and centrifuged bone, respectively, while those for analogous cancellous samples was 186 ($\pm$ 54), 132 ($\pm$ 33) and 28 ($\pm$ 7) ng/ μ L. The average concentration for marrow samples was 191 ($\pm$ 130) ng/ μ L. The 260/280 ratio was within an acceptable range (1.8–2.1) for all samples.

Cancellous samples were of higher quality than their cortical counterparts with a mean ($\pm$ SD) RQN of 6.3 ($\pm$ 0.7) and 4.4 ($\pm$ 0.4), respectively.

3.3 Gene Expression

We examined bone-and marrow-related gene expression in cortical and cancellous bone as well as bone marrow and the effect of bone marrow contamination. Centrifuged cortical bone had 12-and 13-fold higher expression of *Colla1* and *Bglap* than bone with marrow, while the analogous data for centrifuged cancellous bone were 30-fold and 31-fold higher, respectively (Figure 3A/B). Flushing also increased bone-related gene expression when compared to no marrow removal, but this effect was not significant (2-fold and 3-fold, *Colla1* and *Bglap* in cortical and cancellous bone, respectively). The absolute expression of *Colla1* and *Bglap* was lower in cancellous compared to cortical bone. Finally, marrow samples had extremely low expression of bone-related genes, ranging from 71-fold to 930-fold less than bone with marrow.

Centrifugation, but not flushing, decreased the expression of a gene that is highly expressed in bone marrow (Figure 3C). *Icam4* expression in centrifuged cortical and cancellous bone was 3-and 2-fold lower, respectively, than intact bone and similar in both bone tissues. Flushing did not alter *Icam4* expression. Marrow samples and cortical and cancellous bone with marrow had similar expression of *Icam4*.

4. DISCUSSION

We initiated these experiments based on our desire to perform more detailed analysis beyond our well-established tissue-level detailing of changes in cortical and cancellous bone following in vivo loading. Moreover, our recent report on the sexually-dimorphic and tissue-specific responses following loading of mice lacking estrogen receptor- α provided further impetus to pursue cellular-and molecular-based studies [15]. Finally, a review of the literature revealed no publications documenting the goals and techniques described here.

We show that RNA can be successfully isolated from centrifuged cortical and cancellous tissues of the mouse tibia separately and used for gene expression analysis. The ability to measure gene expression separately in cortical and cancellous bone is an important achievement. For example, we discovered that expression of two highly-expressed bone genes varies between the two tissue types. While both methods decreased marrow contamination, centrifugation removed marrow more completely than flushing in both cortical and cancellous sites when compared to intact bone. Quantitatively, this removal of contaminating marrow cells allowed bone transcripts to be more easily detected by qPCR. Bone genes were as much as 31-fold more highly expressed in centrifuged bone, while expression of a marrow gene was decreased as much as 3-fold.

The ability to examine transcriptional activity in cortical and cancellous bone separately from a mouse is a significant achievement that has the potential to impact osteoporosis and mechanotransduction research. Cancellous bone, the major modulator of osteoporosis-associated fragility fractures, may respond differently to mechanical loading than the adjacent cortical component; this subject remains completely unexplored. While several

studies in rodents have examined gene expression in cancellous bone [10, 16, 17], none have compared expression to the cortical bone at the same location in the mouse. Importantly, here cancellous bone from unmanipulated wild type mice had lower expression levels of bone-related genes than cortical bone, demonstrating the importance of examining these sites separately. Moreover, these site-specific differences are likely to vary with age, sex, genetic background or exposure to a wide range of environmental, lifestyle or drug treatment-related stimuli.

Although we have shown that centrifugation is an efficient method of marrow removal, heterogeneous cells are likely still exists in the bone samples: for example, adherent hematopoietic and endothelial cells and a mixture of osteoblasts and osteocytes. Further work should determine the exact cellular makeup of our bone samples, including a marker of endothelial cells, such as CD31. The amount of RNA obtained from these bone sites after centrifugation was relatively small, limiting the number of genes that can be examined with qPCR. However, the RNA was of high quality and is appropriate for gene expression profiling with RNA sequencing, which requires much less input material.

4.1 Conclusions

Cancellous and cortical bone can be isolated separately from the mouse tibia for RNA isolation and used for gene expression studies. In all future studies examining bone gene expression centrifugation should be adopted for removing bone marrow from bone tissue since it is more efficient than flushing. The separate examination of cancellous and cortical bone has the potential to elucidate the differential response to mechanical loading as well as genetic manipulations resulting in different bone phenotypes in these sites.

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Highlights

- Cortical and cancellous bone samples were isolated from mouse tibiae with negligible marrow contamination.
- Centrifugation and flushing to remove marrow were compared with histology and qPCR.
- High quality RNA can be isolated from cortical and cancellous bone separately, allowing gene expression comparison.
- Centrifugation removes marrow more efficiently than flushing and enhances detection of bone specific genes in cortical and cancellous bone.

Figure 1.

Schematic of cortical and cancellous bone sample preparation. Tibiae were rapidly dissected and soft tissues, periosteum, epiphysis, fibula, and distal end were removed. Marrow was left intact (group 1), flushed with 1ml of PBS using a 26g needle inserted distally (group 2), or centrifuged in nested microcentrifuge tubes for 20 seconds at 16,100g (group 3). Tibiae were cut approximately 5mm distal to the proximal end to isolate the metaphysis, and a 1mm biopsy punch mechanically separated a cancellous core from the cortical shell.

Figure 2.

Centrifugation removes marrow more effectively than flushing in cortical and cancellous bone. Representative longitudinal 6 μ m sections of (A-C) cortical and (D-F) cancellous bone stained with H&E of (A,D) bones with no marrow removal, (B,E) bones where bone marrow has been flushed, and (C,F) bones that have been centrifuged to remove marrow. Top row within each bone site is lower magnification and black rectangle outlines region of higher magnification shown on bottom row. Scale bar = 400 μ m

Figure 3.

Centrifugation resulted in higher expression of bone related genes and lower expression of a gene in bone marrow. Top panels show relative expression to GAPDH (2^{-C_T}) and bottom panels are fold-change to NMR group (2^{-C_T}). (A) *Colla1* was 12-and 30-fold more highly expressed in centrifuged cortical and cancellous bone, respectively, when compared to NMR. *Colla1* expression in flushed bone was not significantly different than bone with marrow. Marrow had very low relative expression of *Colla1* that was 932-and 307-fold lower than cortical and cancellous bone with marrow, respectively (values above bar). (B) *Bglap* was 13-and 31-fold more highly expressed in centrifuged cortical and cancellous bone, respectively, when compared to NMR. *Bglap* expression was not significantly changed in flushed bone compared to bone with marrow. Marrow had very low relative expression of *Bglap* that was 261-and 71-fold lower than cortical and cancellous bone with marrow, respectively (values above bar). (C) *Icam4* expression was decreased in centrifuged cortical and cancellous bone when compared to NMR. *Icam4* expression was similar in NMR, flushed bone, and marrow as indicated by dashed line at a fold-change value of one. Marrow had similar *Icam4* expression to bone with marrow. Data are mean $\pm$ SD, $n=3-5$ per group. *Main effect of site: cancellous different from cortical. Brackets indicate main effect of marrow removal: no marrow removal or marrow and flushed different from centrifuged. ^{a-c}Groups with different letters are significantly different by Tukey HSD post-hoc. †Cortical NMR different from cancellous NMR and marrow groups by one-way ANOVA.