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Method for measuring lipid mediators, proteins, and messenger RNAs from a single tissue specimen

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ABSTRACT

This article describes a new method for extracting RNA, protein, and lipid mediators from a single tissue specimen. Specifically, mouse bone fracture callus specimens were extracted into a single solution that was processed using three different procedures to measure messenger RNA (mRNA) levels by reverse transcription–quantitative polymerase chain reaction (RTqPCR), cytokines and growth factors using an xMAP method, and lipid mediators by liquid chromatography–tandem mass spectrometry (LC–MS/MS). This method has several advantages because it decreases the number of animals necessary for experimentation, allows division of the sample from a homogeneous mixture that reduces sample variability, and uses a solution that protects the integrity of the macromolecules during storage.

Bone fractures normally heal by tissue regeneration, which occurs through a temporally and spatially coordinated process [1,2]. Fractures typically disrupt blood circulation, causing hematoma formation and localized tissue hypoxia. Inflammation quickly follows, and lipid mediators, growth factors, and cytokines released at the fracture site promote proliferation and chemotaxis of cells to the site. Mesenchymal cells that have proliferated or migrated to the fracture site differentiate into chondrocytes to form a cartilaginous callus around the fracture. Bone replaces the cartilage by endochondral ossification to bridge the fracture. The newly formed bone is remodeled to increase mechanical strength and restore the shape of the bone. Although the histological processes that occur during fracture healing are well described, the signaling events that control this tissue regeneration process are poorly understood.

Previous studies showed that cyclooxygenase-2 (COX-2)[3] and 5-lipoxygenase (5-LO) activity regulates bone fracture healing. Pharmacological inhibition or genetic ablation of COX-2 impairs healing [3,4], whereas inhibition or ablation of 5-LO accelerates healing [5,6]. COX-2 and 5-LO catalyze the synthesis of prostaglandins and leukotrienes, respectively, which are lipid mediators that regulate inflammation and other processes, including tissue regeneration [7–10]. Measuring the types and levels of lipid mediator during fracture healing is necessary to understand how COX-2 and 5-LO regulate this tissue regeneration process. This is complicated by the altered dynamics of fracture healing caused by loss of COX-2 or 5-LO activity. Thus, levels of each lipid mediator must be correlated to other cellular processes occurring at that time in the fracture callus in order to understand the role each lipid mediator has during fracture healing. Ideally, lipid mediators would be correlated to protein and messenger RNA (mRNA) markers of established physiological and cellular processes in order to understand how bone regeneration is regulated by COX-2 or 5-LO. In general, measurement of mRNA, protein, or lipid mediators is performed with multiple tissue samples prepared separately using extraction

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[3] *Abbreviations used:* COX-2, cyclooxygenase-2; 5-LO, 5-lipoxygenase; mRNA, messenger RNA; RTqPCR, reverse transcription quantitative polymerase chain reaction; LC–MS/MS, liquid chromatography–tandem mass spectrometry; M-PER, Mammalian Protein Extraction Reagent; cDNA, complementary DNA; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Col2a1*, type II collagen; *Acan*, aggrecan; Ct, threshold cycle; SPE, solid-phase extraction; AMP, *N*-(4-aminomethylphenyl)pyridinium; MRM, multiple reaction monitoring; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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methods appropriate for each target molecule [6,11–14]. The spatial complexity of a fracture callus precludes using this approach because dividing the callus into portions would yield tissue samples with different cellular compositions unless specialized methods such as laser capture microdissection were employed [2,15]. Alternatively, a fracture callus could be used to measure one type of target molecule [6,16–18]. However, this approach would require using significantly more animals to measure different types of target molecules and would introduce another level of variability into the analysis because levels of the different target molecule types could not be compared from the same specimen.

To overcome these limitations, we developed methods for isolating lipid mediators, proteins, and RNA from the same tissue specimen. The method relies on RNA*later* solution (Ambion, Austin, TX, USA) to preserve RNA during callus extract preparation and modification of existing methods to measure mRNA, proteins, and lipid mediators from callus extract aliquots using reverse transcription quantitative polymerase chain reaction (RTqPCR), xMAP, and liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods, respectively [19].

Materials and methods

Animal model

Female ICR mice (Taconic Farms, Germantown, NY, USA) weighing 28.7 ± 2.3 g (mean ± standard deviation) were used in this study. Mice were anesthetized by intraperitoneal injection of ketamine and xylazine (0.1 and 0.01 mg/g body weight, respectively). A closed diaphyseal fracture was created in the right femur using a custom-made three-point impactor (BBC Specialty Automotive Center, Linden, NJ, USA) as described previously except that the mice were allowed to recover for 7 days between insertion of the intramedullary pin used to stabilize the fracture and production of the fracture [20]. All experimental procedures were approved by the New Jersey Medical School institutional animal care and use committee.

Tissue collection

On day 4 postfracture, mice were injected intraperitoneally with 0.1 ml of 0.5 mg/ml heparin (USB, Cleveland, OH, USA) 30 min before euthanization to prevent postmortem blood clotting. Femurs were resected with surrounding muscle and callus was left intact. The proximal and distal epiphyses were removed. The final length of femur diaphysis and surrounding tissue was approximately 1 cm and weighed 0.72 ± 0.10 g. The femur was divided at the fracture site to facilitate subsequent tissue homogenization, flash frozen in liquid nitrogen, and stored at −80 °C.

Tissue homogenization

Each femur was homogenized in 3 ml of prechilled RNA*later* that had been spiked with deuterated eicosanoids to act as internal standards. The deuterated eicosanoids were purchased from Cayman Chemicals (Ann Arbor, MI, USA), dissolved in methanol to 500 ng/ml each, and added to a final concentration of 6.67 ng/ml that would ultimately yield a maximum of 2.67 ng of each deuterated eicosanoid per LC–MS/MS injection (see below). Callus extracts were made with a Precellys 24 Dual Tissue Homogenizer (Bertin Technologies, Washington, DC, USA) using 7-ml tubes containing 2.8-mm-diameter zirconium oxide beads. The callus was pulverized twice at 6500 rpm for 15 s. To reduce overheating of the callus extract, samples were placed on dry ice for 1 min before pulverization and were cooled on ice between pulverization steps. After homogenization, 2 ml of the callus extract was immediately processed for eicosanoid analysis and the remainder was stored at −20 °C for protein and RNA analysis.

Protein extraction

Aliquots of the callus extract were dialyzed to remove RNA*later* and solubilize precipitated protein. Protease inhibitors (Sigma–Aldrich P2714 Protease Inhibitor Cocktail) were added to a 0.4-ml aliquot of the callus extract, which was then transferred to a dialysis apparatus (Tube-O-DIALYZER, 1 kDa Micro, G-Biosciences, St. Louis, MO, USA) and dialyzed against M-PER (Mammalian Protein Extraction Reagent, Thermo Fisher Scientific, Rockford, IL, USA) for 24 h at 4 °C. After the dialysis, insoluble material was removed from the samples by centrifugation (14,000 rpm for 10 min at 4 °C). To remove additional debris, the supernatant was collected and filtered through a 0.2-μm membrane by centrifugation (Nanosep, PALL, Port Washington, NY, USA) at 14,000 rpm and 4 °C until all liquid passed through the filter cartridge. The clarified extract was stored at −20 °C. Aliquots of the clarified extract were used to measure total protein content using the BCA (bicinchoninic acid) assay (BCA Protein Assay Reagent, Thermo Fisher Scientific) and to measure target protein amounts using an xMAP method as described below [21,22]. Protein extract quality was assessed by electrophoresis using 8% polyacrylamide Bis–Tris gels in which 15 μg of extract protein was separated in each lane. Protein was detected by Coomassie Blue staining. Precision Plus Protein Standards (Bio-Rad, Berkeley, CA, USA) were used to estimate protein sizes.

xMAP assay

Growth factors and inflammation-related cytokines were measured in the clarified extracts using a Luminex 100 multiplexing instrument (Luminex, Austin, TX, USA) with a Milliplex Map Mouse Cytokine/Chemokine Panel (Millipore, Bedford, MA, USA). A total of 32 target proteins were analyzed (Table 1). Each clarified extract was measured in duplicate using 25 μl of clarified extract for each assay. Mean fluorescence intensity for each target protein was compared with a standard curve developed using standards contained within the Milliplex Map Mouse Cytokine/Chemokine Panel reagents to determine the amount of each target protein within the clarified extract aliquot. The amount of each target protein was then normalized to the total protein amount used in each assay to yield picograms of target protein per milligram of soluble protein.

Total RNA purification

Total RNA was prepared from callus extract aliquots using modified TRIzol extraction and differential filter binding methods in order to eliminate RNA*later* from the purified RNA preparation [13]. Callus extract (100 μl) was combined with 4 volumes of TRIzol and 4 volumes of water. After mixing, another 6 volumes of TRIzol was added and this was mixed gently on a shaker for 30 min at 4 °C. Chloroform (0.1 volumes) was added, mixed, then the aqueous phase was separated by centrifugation (12,000 rpm for 10 min at room temperature), collected into a new tube, and processed by differential filter binding and elution (RNeasy; Qiagen, Valencia, CA, USA) as follows. The aqueous layer from the TRIzol extraction was mixed with 4 volumes of RLT buffer containing β-mercaptoethanol and 3.5 volumes of methanol. This solution was applied to an RNeasy minicolumn. Afterward, the column was washed with 2 volumes of RW1 buffer and 2 volumes of RPE buffer containing methanol. The RNA was eluted with two washes of 25 μl water. RNA concentration was determined spectrophotometrically with a NanoDrop 1000 (Wilmington, DE, USA). RNA integrity