INTRODUCTION: Over 10% of the 5.6 million annual fractures reported in the United States show delayed healing and nonunion. The latter incurs an immense social and economic cost to society. Low-intensity pulsed ultrasound (LIPUS) has been shown to accelerate the rate of fracture healing in animal and human studies. Although the exact mechanism remains unknown, various signaling mechanisms have been implicated in modulating the biological effect of LIPUS. Critical members of these signaling systems, both at the gene and protein level, have been identified in vitro studies. In an effort to more clearly elucidate the timing and expression levels of key proteins previously shown to have influence on the speed and quality of fracture resolution, we performed a detailed, in vivo analysis of MMP-13, Osteopontin, and Cox-2 protein expression in hypertrophic chondrocytes of the fracture callus. The analysis was performed on callus retrieved from femora of rats that were exposed to LIPUS treatment and compared to the contralateral non-exposed side.

METHODS: Animal protocol 43 male Wistar rats, age 53-56 days, underwent bilateral femoral intramedullary fixation with 1x25 mm titanium alloy rods under anesthesia. A closed transverse mid-diaphyseal fracture was produced by the Bonnarens and Einhorn fracture protocol. The fixation and fracture were confirmed by radiography. Following fracture, all rats received 10 minutes of LIPUS (Exogen, Smith and Nephew) to one fracture side, daily for up to 7, 14, 21 days, with the opposite side receiving sham (transducer without LIPUS). At harvest, the animals were sacrificed with CO2, the entire femora were carefully dissected preserving the calluses, and fixed in 4% parafomaldehyde, decalcified, embedded in paraffin and sectioned. The treatment protocols, appropriate anesthesia, and pain control had been approved by the Institutional Animal Care and Use Committee. MMP-13, Osteopontin, and Cox-2 proteins were localized by immunohistochemistry using the DAB method of detection (fig. 1A) in areas of endochondral ossification characterized by hypertrophic chondrocytes. Matched sections of the fracture callus' from each leg of the rat were placed on the same slide, immunohistochemically reacted, imaged and automated analysis performed. The analysis counted the number of positively stained cells within 2 size ranges (fig. 1B) and quantitated the intensity of the staining in each group. This analysis was performed with at least three rats for each time point, at least 8 microscopic fields were acquired with the same settings and exposure time and from multiple sections of the bilateral rat callus'. The images were acquired on a 12bit cooled digital camera (QImaging, Burnaby, BC) as monochrome images. The images were inverted and background intensity subtracted before analysis with ImagePro Plus (MediaCybernetics, Silver Spring, MD).

RESULTS: Immunohistochemical evaluation of the hypertrophic chondrocytes of the fracture callus revealed significant changes in the timing and number of cells expressing MMP-13, Osteopontin, and Cox-2 proteins. These proteins have been identified as key mediators that are required for the resolution of endochondral ossification. Our analysis shows increases in the number of positively stained chondrocytes in ultrasound treated fractures. The number of MMP-13 and Cox-2 positive cells are increased over 100% at week one while osteopontin shows a delayed response increasing at week 2. MMP-13 decreases steadily over time maintaining higher numbers over sham where Cox-2 and osteopontin levels decrease to numbers below sham possibly indicating quicker resolution of fracture healing.

DISCUSSION: The process of fracture healing has been delineated into multiple stages. Endochondral ossification is an intricate process involving development of a cartilage anlage which eventually becomes calcified and is replaced by bone. LIPUS is believed to influence the formation of soft callus and the process of endochondral ossification. MMP-13, Osteopontin, and Cox-2 protein expression are all factors that have been reported to be necessary for the successful resolution of the endochondral transition to bone. MMP-13 expression has been shown to be necessary for breaking down the extracellular matrix in order for VEGF-mediated angiogenesis to take place. Cox-2 is necessary for normal endochondral ossification during fracture healing and osteopontin regulates calcification in the matrix proper of mineralized tissues. Our analysis demonstrates that LIPUS promotes changes in the timing of expression and in the number of cells expressing these proteins. The findings are suggestive of enhanced 'maturity' of the callus or what is referred to as the left-shift in healing. Although the exact mechanism by which LIPUS imparts its stimulatory effect on fracture callus is still unknown, the findings of this and other studies suggest that LIPUS triggers a series of biological events that lead to faster healing of long-bone fractures.

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