Naproxen impairs load-induced bone formation, reduces bone toughness, and delays stress fracture repair in mice

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INTRODUCTION: Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly consumed medication in the world, with over 30 million daily users. Regular NSAID users include as many as 50% of patients over the age of 65 and 80% of active duty US military. NSAIDs are effective in reducing pain and inflammation by preventing the synthesis of prostaglandin E2 (PGE2) through the blockade of the cyclooxygenase (COX) enzyme isoforms, COX1 and COX2. However, the synthesis of prostaglandins is part of the inflammatory signaling necessary for optimal strain adaptive bone remodeling, in which bone is formed in response to mechanical forces at the sites of highest strain. This process has been shown to greatly increase the fatigue strength of bone. Therefore, we reasoned that regular use of NSAIDs in periods of repetitive mechanical loading may result in an increased risk of fatigue injuries, such as stress fracture.

In this study, we investigated the effects of two popular NSAIDs, aspirin and naproxen, on load-induced bone formation and stress fracture repair.

METHODS: All procedures were approved by the Thomas Jefferson University IACUC. Female C57BL/6j mice (Jackson Labs #000664) were obtained at 15 weeks of age, allowed to acclimate for one week, then randomly assigned to receive aspirin (100 mg/kg/d), naproxen sodium (10.9 mg/kg/d), or vehicle in drinking water. Loading. Axial forelimb compression was performed using a material testing system (Restechforce 3200). One group of mice received six bouts over two weeks of a 2 Hz rest-inserted sinusoidal waveform with a peak force of 3.0 N applied for 100 cycles. A separate group received a preclinical stress fracture generated using a cyclic sinusoidal waveform of 3.6 N at 2 Hz applied until 0.84 mm displacement relative to the 10th cycle. Dynamic histomorphometry. Calcein (10 mg/kg) and alizarin red (30 mg/kg) were administered 3 and 10 days after loading, then analyzed in polished PMMA sections. MicroCT. Bones were scanned using a Bruker Skyscan 1275 microCT system (1 mm Al filter, 55 kV, 181 μA, 8 μm voxel, 78 ms exposure). Three-point bending. Standard three-point bending was performed with a span of 8.2 mm. Notched three-point bending was performed with a posterior surface notch. Hydroxyproline. Decalcified tibial samples were delipidized, lyophilized, and hydrolyzed before analysis in triplicate. Collagen crosslinking. Decalcified tibial samples were homogenized in 0.5 M acetic acid on ice, then incubated at 4 °C for 24 hours with pepsin before running on a 6% separating gel. Picrosirius red and SHG imaging. Decalcified sections were stained using picrosirius red, imaged with a polarized light microscope, and analyzed by color thresholding.

RESULTS: Axial forelimb compression produced the expected anabolic response in loaded limbs from vehicle-treated mice. Administration of aspirin did not significantly affect bone formation parameters. However, mice treated with naproxen had significantly decreased relative (loaded – non-loaded) periosteal bone formation parameters, including decreases in rPs.MS/BS (-65%), rPs.MAR (-77%), and rPs.BF/BS (-76%). Surprisingly, we observed a large and significant reduction in bone toughness, which was mainly due to decreased (-48%) post-yield energy. After accounting for minimal differences in geometry, we still observed a significant decrease (-35%) in toughness in bones from mice treated with naproxen as compared to vehicle, which was mainly due to decreased (-45%) post-yield toughness. The effect of aspirin on either energy or toughness was not statistically significant. We also performed notched three-point bending but observed no difference in the critical stress intensity factor (Kc), a measure of resistance to crack initiation at a dominant flaw. We next analyzed collagen content and structure in the hindlimbs of mice treated with aspirin or naproxen (Fig. 1). First, femoral midshaft cross-sections stained with picrosirius red were visualized using polarized light. Color thresholds determined collagen fibril thickness, with quantification of thin (green), medium (yellow), and thick (red) collagen fibrils. The percentage of thin (green) fibrils was significantly increased (+20%) in the naproxen treated mice compared to vehicle. This increase in thinner fibrils was at the expense of thick (red) fibrils, which were significantly decreased (-49%) in naproxen treated mice. There were no significant differences in aspirin treated mice. Consistent with these results, we found a significant decrease in SHG signal in bones from mice treated with naproxen (-32%), but there was no effect of aspirin. We also observed that treatment with either aspirin or naproxen significantly decreased collagen content in the tibia, as quantified using a colorimetric assay for collagen-specific 4-hydroxyproline. However, no differences in the crosslinking of the collagen fractions extracted from tibial samples were observed between groups by SDS-PAGE. In a separate experiment, mice were subjected to an ulnar stress fracture generated by a single bout of fatigue loading at 16 weeks of age. We observed that vehicle-treated mice used their loaded forelimb significantly less (-25% to -34%) on days 3 to 6 as compared to baseline. In contrast, there was no significant difference in forelimb usage in mice treated with either aspirin or naproxen at any time point. However, the amount of woven bone formed 7 days after stress fracture was significantly diminished (-27%) in bones harvested from mice treated with naproxen, but not aspirin. Furthermore, Collagen crosslinking was significantly diminished by both NSAIDs at 3 hours and 7 days, whereas Ngf expression was significantly diminished by both NSAIDs at 3 hours only. We did not observe increased Ngf expression in any group 5 days after injury.

DISCUSSION: In this study, we analyzed the effects of two popular NSAIDs, aspirin and naproxen, on load-induced bone formation and stress fracture repair in mice. Our main objectives were to determine if these drugs would increase the risk of stress fracture during periods of intense physical activity or impair the healing process following a fatigue injury. In total, our results indicate that naproxen, but not aspirin, was associated with a significant decrease in load-induced bone formation and an unexpected loss of bone toughness over a period of two weeks of loading. The decrease in toughness was nearly exclusively due to diminished post-yield deformation, which we attribute to alterations in bone collagen structure and organization. However, we did not analyze other factors that could alter bone toughness, such as hydration, GAGs, or direct drug-bone interaction. We also noted that naproxen, but not aspirin, was associated with diminished woven bone formation following stress fracture, although both relieved stress fracture-related pain equally well. More study into the specific mechanism of action in bone downstream of aspirin and its metabolites may reveal new therapeutic targets for relieving bone pain without negatively affecting the skeleton. In total, we conclude that the routine use of naproxen may increase the risk of stress fracture in active individuals and/or extend the time required for healing, and therefore warrants further clinical investigation and caution use in subjects with routine intense physical activities.

SIGNIFICANCE: Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs in the world, but their effect on bone health and healing is not well understood. In total, our results suggest that naproxen, but not aspirin, should be avoided by individuals with intense physical routines due to the increased risk of fatigue fracture and diminished repair of skeletal injuries.

Figure 1. A-C) Picrosirius red stained sections under polarized light and D-F) Second harmonic generation (SHG) imaging were G,H) quantified to show altered collagen fibril size and structure in bones from mice treated with naproxen.

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