Complex I Inhibition after Intra-articular Fracture Prevents Rapid Progression of Osteoarthritis in a Porcine Model

Mitchell C. Coleman[1], Jessica E. Goetz[1], Marc J. Brouillette[1], Michael C. Willey[1], Dongrim Seol[1], Emily B. Petersen[1], Behnoush Khorsand[1], Aliasger K. Salem[1], Douglas C. Fredericks[1], Todd O. McKinley[2], James A. Martin[1]
[1]University of Iowa, Iowa City, IA [2]Indiana University, Indianapolis, IN

Disclosures: Mitchell C. Coleman(N), Jessica E. Goetz(N), Marc J. Brouillette(N), Michael C. Willey(N), Dongrim Seol(N), Emily B. Petersen(N), Behnoush Khorsand(N), Aliasger K. Salem(N), Douglas C. Fredericks(N), Todd O. McKinley(N), James A. Martin(N).

INTRODUCTION: The rapid progression of PTOA after severe injuries like intra-articular fractures (IAF) combined with a lack of therapeutic options other than total joint replacement at end-stage suggests a need for new treatment paradigms to avoid or delay PTOA progression (1). Our previous *in vitro* studies have demonstrated protection of chondrocytes after impact or high strain using rotenone, a well characterized irreversible inhibitor of complex I of the mitochondrial electron transport chain (2, 3). Similar protective effects with the non-specific thiol antioxidant N-acetylcysteine (NAC) suggested that thiol oxidation and oxidative stress could also mediate injury in this pathway (4). This study extends our line of experimentation using a maximally clinically realistic IAF model (5) and the barbiturate amobarbital in place of rotenone. Amobarbital represents a more therapeutically viable inhibitor of complex I than rotenone given its reversible binding to complex I and decreased toxicity (6). **Hypothesis:** Administration of amobarbital or NAC subsequent to IAF and surgical fixation will delay the rapid progression of PTOA.

METHODS: Our porcine model utilizes a 40 J impact to the talus to cause a reproducible distal tibial fracture without surgical disruption of the joint capsule (4). Fractures are repaired using human-like open reduction and internal fixation (ORIF). PTOA occurs by six months, with lesions concentrated around the central-to-anterior portion of the medial talus. Either 2.5 mM amobarbital or 10 mM NAC (buffered with sodium bicarbonate) was dissolved in F-127/hyaluronic acid reverse thermal hydrogel. ORIF with vehicle alone (n = 5), amobarbital (n = 5), or NAC (n = 6) hydrogel was injected intra-articularly immediately after completion of the ORIF and again one week later. In addition to the injection, some NAC animals received placement of a small grain of poly(lactic-co-glycolic) acid (PLGA) encapsulated NAC for extended release over ~2 weeks after injury; however, we have pooled all NAC-receiving animals after observing no differences with the extended release pellet. Sham animals (n=5) receiving surgical procedures without fracture impact as well as naïve controls (Normal - n=5) were included. After treatment, animals were put into pasture for the duration of six months, after which they were euthanized. All procedures were conducted under IACUC approved protocols. Fractured and intact hocks were harvested and small pieces of cartilage were taken for live cell respiratory analyses. The remaining distal tibia and talar dome were fixed in formalin, decalcified and embedded in paraffin. Sagittal sections of weight-bearing tissue 5 µm-thick were cut and stained with safranin O, fast green, and Weigert's hematoxylin (5). Semi-automated Mankin scoring of these sections used a custom Matlab program, which quantified joint histology as previously described (5). Treatment effects were analyzed by one-way ANOVA.

Confirmation of inhibition of complex I by amobarbital was conducted *in vitro* using fresh harvested bovine chondrocytes (7). Briefly, cell lysate is added to phosphate buffer pH 7.2 with excess NADH, CoQ and complex III blockade while oxidation of NADH is monitored at 340 nm for 3 minutes with and without rotenone. This yields a rate of rotenone-inhibitable NADH oxidation, i.e. complex I activity. Complexes II, III, and IV were assessed with similar methods (7) but with no observable inhibition by amobarbital. We also conducted extracellular flux measurements via Agilent Seahorse XF96 in the presence and absence of 2.5 mM amobarbital, confirming > 90% decreases in oxygen consumption. In order to confirm the antioxidant effects of NAC one week after porcine IAF, steady states of the glutathione (GSH):glutathione disulfide (GSSG) redox couple were assessed as described (8).

RESULTS: Fresh whole chondrocyte lysates demonstrated a dose responsive inhibition of complex I by amobarbital, reaching >90% at maximal doses (**Figure 1A**, $p < 0.01$ at 2.5 mM, n = 3). Porcine tissue harvested 1 week after IAF demonstrated increased steady state percentages of GSSG, indicating oxidative stress, that were not present after NAC treatment (**Figure 1B**, $p < 0.01$ for Hydrogel v NAC, n = 6). These results support the hypotheses that amobarbital and NAC suppress complex I activity and oxidative stress, respectively. Sham animals displayed no increases in Mankin scores (**Figure 2A**) while fracture plus ORIF induced a significant increase in overall Mankin score (**Figure 2A**), decreased overall cartilage thickness and safranin O staining, and caused focal areas of eburnation (**Figure 2B,** representative micrograph). Animals receiving amobarbital after ORIF (ORIF+Amo) or NAC after ORIF (ORIF+NAC) showed statistically significant decreases in semi-automated Mankin scoring (**Figure 2A**, $p < 0.01$ for amobarbital, $p = 0.0322$ for NAC). These animals also clearly demonstrate thicker tissue and stronger staining for safranin O (**Figure 2B**, representative micrographs).

DISCUSSION: These results demonstrate that manipulation of joint mitochondrial metabolism after traumatic injury represents a viable pathway to treating PTOA. We have confirmed that the doses of amobarbital used *in vivo* provide inhibition of complex I directly. NAC at these doses and in comparable IAF pigs at one week provided protection against intracellular thiol oxidation, supporting the hypothesis that thiol-mediated oxidative stress plays a role in responding to the mitochondrial oxidant production inhibited by amobarbital. It is important to note that no porcine *in vivo* dose optimization or timing optimization studies have been done, but preliminary experiments in a rabbit impact model suggest the window for amobarbital treatment is likely between 4 and 8 hours after injury which represents a realistic window in which to provide a patient with an intra-articular therapy. We also have not yet combined this approach with any more specific anti-inflammatory approaches that may augment any efficacy.

SIGNIFICANCE: These results support the hypothesis that a therapeutic window for blunting PTOA exists at the earliest stages of disease. Given that NAC and amobarbital have such disparate effects upon cells but similar benefits for disease, these data also strongly support the importance of mitochondrial oxidant production and responsive intracellular thiol pathways in PTOA initiation after mechanical injury.

REFERENCES: 1. McKinley TO, et al. J Orthop Trauma; 24(9):567-70. 2. Wolff KJ, et al, J Orthop Res; 31(2):191-6. 3. Brouillette MJ, et al. Biomech Model Mechanobiol; 13(3):565-72. 4. Martin JA, et al. J Bone Joint Surg Am; 91(8):1890-7. 5. Goetz JE, et al. Osteoarthritis Cartilage. 23(10):1797-805. 6. Harris EJ, et al. Biochemistry; 6(5):1348-60. 7. Birch-Machin MA, et al. Biochem Med Metab Biol; 51:35-42. 8. Griffith OW. Anal Biochem; 106:207-12.

ACKNOWLEDGEMENTS: This work was funded by DOD #W81XWH-11-1-0583 and NIH CORT: 5 P50 AR055533-09.

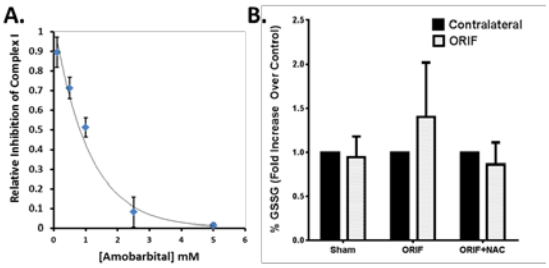

Figure 1. Amobarbital Inhibits Complex I Activity and NAC Prevents Intracellular Thiol Oxidation after IAF

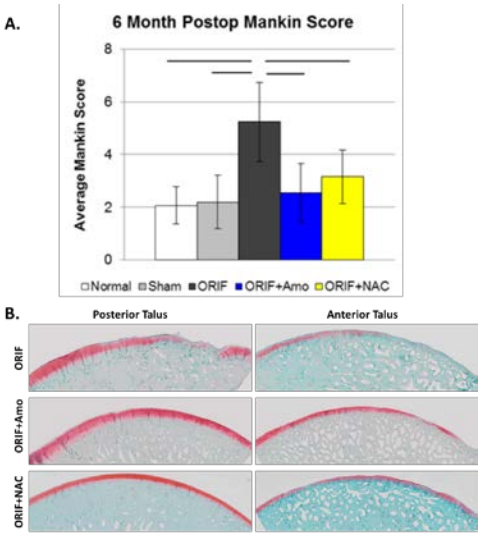

Figure 2. Amobarbital and NAC Prevent Rapid PTOA Progression Six Months Post-IAF