An Immunoregenerative Approach to Mitigating Post-Traumatic Osteoarthritis in Rats

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Introduction: Intra-articular fractures (IAF) lead to significant inflammation and joint misalignment, posing challenges to the healing of the bone and cartilage, and significantly raising the risk of post-traumatic osteoarthritis (PTOA) compared to injuries that don't reach the subchondral bone. Inflammatory cytokines released by immune cells (infiltrating leukocytes) play a crucial role in the development of PTOA. While we and others have found that blocking these cytokines using targeted biologics can reduce the impact of PTOA, it doesn't fully reverse the damage. At the same time, there are promising developments in using substances that encourage cartilage regeneration on the joint surface. Kartogenin (KGN) and its structural analogue KA9 promote the transformation of mesenchymal stem cells into chondrocytes via nuclear translation of transcription factor RUNX1, which leads to the production of Collagen 2 (Col II) and aggrecan, essential components of healthy cartilage. With these insights, we hypothesized that treating both the inflammatory environment and the direct damage to the bone and cartilage through a combined immunoregenerative approach would enhance the healing process and reduce the development of PTOA after traumatic joint injuries. Therefore, the objective of this study was to investigate the effects of using KGN and KA9 in conjunction with Anakinra (ANR), an IL1 receptor antagonist, on the progression of PTOA following IAF.

Methods: This study was approved by the institutional animal care and use committee at the Uniformed Services University of the Health Sciences. Skeletally mature male Lewis rats (325-350g; n=6/group) were subject to unilateral IAF via a 5J blunt impact to the lateral aspect of the knee. Immediately following injury, an osmotic pump (Alzet) loaded with either saline or ANR at 100µg/kg/day were implanted subcutaneously and delivered agents continuously for 14 days. Following IAF and pump implantation, injured knees were subject to injections with 1µg of KGN or KA9 suspended in 100µl of saline (also used as injection control). Injections were performed weekly for 6 weeks. Body weights (BW) were recorded, and blood was drawn one day prior to injury and at 14- and 56-days post-injury (DPI). Following euthanasia (14 and 56 DPI), livers and kidneys were collected for toxicity assessments. Serum and SF were harvested for soluble factor profiling (ProcartaPlex Rat 22 Cytokine/Chemokine Array, Invitrogen) and assessment of markers of osteochondral remodeling. Limbs were harvested for histopathological analyses. Micro-computed tomography (µCT) was performed at terminal endpoints to assess IAF fracture healing through changes in bone mineral density (BMD) and bone morphometry measurements (BMM). Iodated cationic agent $CA4^{+}$ was used to measure sulfated glycosaminoglycans (sGAGs) via ex vivo contrast enhanced µCT (CE-µCT). Following CE-µCT, tibias were processed for paraffin embedding, section, stained for H&E and Safranin O, and scored using a modified OARSI methodology. Additionally, tissues were IHC stained for macrophages/inflammation, cartilage and bone degradation with antibodies targeting CD68, iNOS, COL2 and DCSTAMP, respectively. BMD, BMM and inflammatory markers were analyzed by ANOVA with Šidák post-hoc tests and are reported as the mean± SEM with significance level set at α=0.05.

Results: No indications of systemic toxicity were observed with our treatment as BW, organ weights (liver and kidney), and organ histology showed no difference relative to saline controls. Evaluation of µCT scans immediately following injury revealed that all tibias incurred IAF and at similar intensity and classification between groups. Evaluation of local inflammatory markers at D14 revealed that compared Sal/Sal, Sal+KA9 and ANR+KGN reduced IL-1α, each by nearly 50% ($P<0.01$). KGN and KA9 alone (i.e. not with ANR) reduced IL-10, each by nearly 50% ($P<0.05$). Finally, ANR+KGN increased IL-17 compared to Sal/KGN control ($P<0.05$). Local expression of osteochondral markers at D56 showed KGN alone increased NTX1 by nearly double ($P<0.05$) but was reduced with addition of ANR back to control levels. Systemic expression of osteochondral markers revealed that compared to Sal/Sal; KGN, KA9 and the combination each with ANR significantly ($P<0.05$ each) reduced expression of NTX1, CTXI and CTXII. COMP was not reduced. BMM analysis revealed that KGN and KA9 had reduced bone surface, surface to volume, connectivity density, object number and degree of anisotropy compared to Sal/ Sal controls, but with increased in trabecular thickness and bone mineral density ($P<0.05$, each). Cartilage analysis via CE-µCT revealed no change in overall distribution of stain intensity between groups, but an increase in overall cartilage density in both the lateral and medial aspects of animals treated with ANR+KGN ($P<0.05$ each). Cartilage density was increased in nearly all treatment groups compared to Sal/Sal control ($P<0.05$ each) in both medial and lateral aspects, except KA9 alone. Finally, cartilage thickness was increased with each treatment ($P<0.05$ each). H&E and Saf-O staining was used for OARSI scores at D56 and revealed no differences between groups but difference at location (i.e. medial vs lateral, $P<0.01$). IHC stains revealed differences in protein expression between groups at D14 and D56 post-injury looking at markers including DCSTAMP, CD68, iNOS and COL2.

Discussion: The current study tested the hypothesis that osteochondral protective agents KGN, and a chemically similar test molecule KA9, would protect against PTOA development by helping to maintain or restore cartilage and subchondral bone health. Moreover, these protective effects would be enhanced by addition of ANR. These results presented here support this hypothesis. KGN and KA9 each reduced osteochondral breakdown markers COMP, NTX1 and CTX1 measured systemically. Additionally, combinatorial therapies using ANR+KA9 (and to a lesser extent ANR+KNG) were also shown to increase cartilage thickness, density and diameter compared to untreated IAF injured animals. Bone mineral density was also slightly increased with each treatment, with the dual treatments having the most significant effect. Interestingly, however, there was little to no impact from mono or dual therapies on the local inflammatory response, BMM or histopathology scores for PTOA. Despite not modify all parameters of PTOA pathology, the overarching effect on cartilage tissue and osteochondral markers suggest that a dual therapeutic approach for treatment of IAF, namely ANR+KA9, may be effective for damping progression to PTOA. While these results are positive, they are generally subtle in nature given the severity of the IAF model and the challenging testbed it represents. It is plausible that the effect of our intervention could be more pronounced for less severe joint injuries. Future studies will address the local effects of these treatments on synovial fluid composition as well as a deeper look into articular surface composition and bone morphometry.

Significance/Clinical Relevance: This study provides meaningful evidence for the use of clinically available drugs to potentially treat IAF leading to PTOA. The results from this study help to provide justification to utilize combinatorial, multi-treatment approach to clinically treat IAF that might lead to PTOA.