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14. ABSTRACT: Since it is known that cartilage catabolic enzymes in synovial fluid (SF) play a critical role in the onset and progression of post traumatic osteoarthritis (PTOA), the objective of this study is to demonstrate that intra-articular injection of α-2 Macroglobulin (A2M), a master inhibitor of these enzymes, would serve as an early therapeutic agent to prevent PTOA after joint injury. In this proposed study, we will test whether early supplemental intra-articular A2M injections will attenuate PTOA pathogenesis 15 weeks after joint injury in the mini-pig model in vivo and we will explore the mechanism how A2M prevents PTOA via blockage IL-1/NF-kb pathway in vitro. In chondroctes culture, we found A2M labeled with VivoTag^{TM} 680 was easy detected by fluorescence microscopy, which indicated that A2M was able to enter into the cells. Western blotting and immunoprecipitation(IP) experiments further showed that A2M was able to bind to IL-1β and inhibited IL-1β and NF-κB in a dose dependent manner. IL-1β induced the expression of NF-κB, while A2M reduced the level of NF-κB induced by IL-1β. These findings indicated that A2M inhibits inflammation via binding to and inhibiting IL-1β, which blocks NF-κB pathway in the chondrocytes. We just performed 8 pigs surgery on August 27-31 in vivo and will collect and analysis the cartilage 15-weeks post-op (the beginning of next year). Successful implementation of an injectable therapeutic to prevent the PTOA has the potential to significantly improve the quality of life for thousands of wounded warriors, as well as maximizing their function for return to duty or civilian life.

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1. **Introduction:** Since it is known that cartilage catabolic enzymes in synovial fluid (SF) play a critical role in the onset and progression of post traumatic osteoarthritis (PTOA), the objective of this study is to demonstrate that intra-articular injection of α-2 Macroglobulin (A2M), a master inhibitor of these enzymes, would serve as an early therapeutic agent to prevent PTOA after joint injury. The mechanisms of PTOA following joint injury are likely due to the biological insults at the time of injury. Anterior cruciate ligament (ACL) injury is one of the most common injuries in the young and military population and is known to place the patient at risk for PTOA. Traditionally, these injuries are surgically treated with ACL reconstruction (ACL-R) to restore biomechanical stability to the joint. However, even with our best current surgical techniques, these patients still remain at high risk for PTOA. The dramatic increase in SF catabolic enzymes following joint injury initiates PTOA. Therefore, early intervention to eliminate catabolic enzymes may be critical for the prevention of PTOA. In this study, we will test **1) l**imiting catabolic enzymes through the intra-articular injection of A2M immediately following injury will reduce the microscopic cartilage integrity score (OARSI) and catabolic enzyme concentrations in the A2M treated animals compared to the no-A2M treated control; 2) the vertical ground reaction forces between limbs will return to pre-operative values early in the injury A2M treated animals compared with injury (no A2M) treated animals; 3) we will explore the mechanism in which how A2M prevents PTOA using cell culture. Successful implementation of an injectable therapeutic to prevent the PTOA has the potential to significantly improve the quality of life for thousands of wounded warriors, as well as maximizing their function for return to duty or civilian life.
2. **Keywords:** Post traumatic osteoarthritis (PTOA); cartilage; joint injury; inflammation; therapy; α-2 Macroglobulin (A2M); knee joint; Anterior cruciate ligament (ACL)
3. **Accomplishments:** Brown University closed whole campus labs due to COVID-19 pandemic and reopened the campus labs conditionally July 2020. Our animal project was reapproved by Brown on July 6th, 2020.
   3-1. **Major goals of the project.** We have three goals for this project: **1) l**imiting catabolic enzymes through the intra-articular injection of A2M immediately following injury will reduce the microscopic cartilage integrity score (OARSI) and catabolic enzyme concentrations in the A2M treated animals compared to the no-A2M treated control; 2) the vertical ground reaction forces between limbs will return to pre-operative values early in the injury A2M treated animals compared with injury (no A2M) treated animals; 3) we will explore the mechanism in which how A2M prevents PTOA using cell culture.
   3-2. **Accomplishments. 3-2-1**. We have been practicing the surgical procedure in order to success perform the surgery using pig carcass specimen collected from residence clinical training (N=10). We also did synovial fluid collection training (N=2) using the pig knee specimen. **3-2-2 (major goal 1)**. In

vivo study, we have performed 8 live pigs surgery on August 27-28, 2020. We will collect and analyze the knee cartilage 15-weeks post-surgery (at the beginning of next year). **3-2-3 (major goal 2)**. We will collect the gait data from the 8 pigs after surgery at the different time points until the 8 pigs are sacrificed 15 weeks post-surgery. **3-2-4 (major goal 3)**. In vitro study, we isolated chondrocytes from pig knee (N=2) for the mechanism study. We also used human chondrocyte cell line (C28) to validate the pig cell result because the human chondrocytes are more relevant to human osteoarthritis. We found that the antibody IL-1β (cell signaling) interacted with IL-1β protein in both pig chondrocytes and human chondrocytes but the NF-kB antibody (cell signaling) only interacted with NF-kB protein in the human chondrocytes and did not interact with pig chondrocytes. We searched the internet and could not find any NF-kB antibody that can interact with pig chondrocytes. NF-kB is a key target for the mechanism study. Therefore, we decide using human chondrocytes for the mechanism study. We found that In C28 cell line, A2M labeled with VivoTag^{TM} 680 was easy detected by fluorescence microscopy, which indicated that A2M was able to enter into the cells (Figure 1). Western blotting and IP experiments further showed that A2M was able to bind to IL-1β and inhibited IL-1β (Figure 2) and NF-κB in a dose dependent manner (Figure 3). IL-1β induced the expression of NF-κB, while A2M reduced the level of NF-κB induced by IL-1β. These findings indicated that A2M inhibits inflammation via binding to and inhibiting IL-1β, which blocked NF-κB pathway in C28 human chondrocytes. **3-2-5**. One abstract entitled "Alpha2-macroglobulin inhibits chondrocyte catabolism by blocking IL-1β/NFkB pathway" has been submitted to 2021 ORS （appendix 1）.

3-3. **Training and professional development.** Nothing to report.

3-4. **The results disseminated to communities of interest.** Nothing to report.

3-5. **Plan to do during the next reporting period.** We only can handle 8-10 pigs each time due to huge amount work and the pandemic. The first 8 pigs will be sacrificed at the beginning of the next year (need 17-18 weeks for each group animals). We will perform gait data collection and synovial fluid collection during the period. We will analyze the cartilage changes from 8 pigs by histology after the animals are sacrificed. We plan to perform another 20 pigs in next reporting period. We will continue to finish the mechanism study using cell culture.

4. **Impact:**

4-1. Impact on the development of the principal disciplined of the project. Nothing to report.

4-2. **Impact on other disciplines.** Nothing to report.

4-3. **Impact on technology transfer.** Nothing to report.

4-4. **Impact on society beyond science and technology.** Nothing to report.

5. Changes/problems:

5-1. **Changes in approach and reasons for changes.** We requested to modify the surgical procedure originally proposed in the grant to improve model performance and animal welfare. The request has been approved by both Brown IACUC and ACURO. The reasons for the request were: In getting ready to begin the study, my co-investigators, Drs Owens, Beveridge and Fleming, and I have been practicing the surgical procedure to core out the femoral insertion of the ACL in cadavers. The coring procedure was originally developed to represent an ideal ACL graft as the surgical trauma is induced while minimizing damage to the ACL (O'Brien et al: J Orthop Res 2013). In doing these practice trials, we found that the coring out procedure induces additional damage to the joint, such as cutting some of the fibers of the normal ACL and introducing cartilage damage. In brainstorming better ways to improve the model, we came up with a modification to the coring procedure originally described in the grant. We propose drilling two 2mm osseous tunnels through the tibia and femur that are adjacent to the intra-articular tibial and femoral insertions of the ACL. The modified procedure will still simulate the effects of the drilling required to implant a graft, which biologically induces cartilage damage, without the risk of mechanically damaging the ACL or articular cartilage. Unlike the coring model, which is only performed at the femoral insertion, we would perform the modified procedure on both the tibia and femur, which is more representative of the drilling required during ACL reconstruction surgery. Furthermore, the drilling model has been previously used to surgically induce post-traumatic osteoarthritis in similar animal models (Huebner et al: J Orthop Res 2013) so we know it will have the same effect. As the modified procedure will induce less damage to the joint and take less OR time, it will be easier on the animals. Furthermore, we know that with the original coring procedure, there are times when the insertion will be

violated so that the animal will need to be excluded from the study. Thus, from an animal welfare perspective, the new procedure will be optimal.

Aim 2 is to measure gait changes between the A2M-treated animals and the sham treated animals before surgery and at 15 weeks after surgery. Just prior to running the first group of 8 animals, we learned that the gait walkway had gotten wet when we were calibrating the system. Due to the water damage, the walkway is not functioning. While this problem most likely can be fixed, the manufacturer is requiring us to ship it back to the company for analysis and repair. This may take a few weeks. Unfortunately, the animals are in house and scheduled for surgery starting on the 27th of August. However, the cost of housing the animals while waiting for the walkway to be repaired would be too much for the budget. Thus, we are proposing to not measure the gait pre-operatively in the first 8 animals of the study. I do not think this will be a serious concern as we will have the system up and running to get the more important 15 week post-operative assessment, in which we will still be able to make comparison of the gait parameters between the A2M and sham treated animals at that time point. We intend to perform the pre- and post-operative measures on the subsequent groups of animals for this study.

In the project, our priority goal is to inhibit the catabolic enzymes by A2M in synovial fluid (SF) because the SF is a main nutrition source for cartilage. However, the inhibition of the catabolic enzymes presented in cartilage might have some benefits. Therefore, we proposed adding lipid nanoparticle (LNP) delivery system that may help A2M diffusing into cartilage to inhibiting the catabolic enzymes presented in cartilage. The LNP was provided by Dr. Ge Zhang from Hong Kong Baptist University in our previous study. Unfortunately, they can't provide LNP for our large animal study. Therefore, the approved IACUC and ACURO don't include LNP delivery system. It should be noted that the modified procedure will not change the overall experimental design or the cost of the study.

5-2. **Actual or anticipated problems or delays and actions or plans to resolve them.** The project may delay due to the pandemic. Whole brown labs just reopened on July, 2020. We plan to accelerate the process by performing 20 animals in the second year and 20 animals in the third year. If we have problem to finish the project, we will request one year no cost extension.

5-3. **Changes that had a significant impact on expenditures**. Nothing to report.

5-4. **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**. Nothing to report.

6. **Products:** Nothing to report.
7. **Participants & other collaborating organization:**

7-1. Individual worked on the project (at least one person month per year)

Name	Project role	Research Identifier (e.g. ORCID ID)	Nearest person month worked	Contribution to project	Funding support
Lei Wei	PI	https://orcid.org/0000-0003-0352-1517	6	Participate in the experiment and Monitor the process. Writing report	
Braden Fleming	Co-PI	https://orcid.org/0000-0002-7841-425X	1.2	Surgery, gait analysis, interpret data, writing	
Brett Owens	Co-PI	0000-0002-9972-0096	1.2	Surgery, interpret data, writing	
Changqi Sun	Research associate	N/A	12	Perform animal study and collect data	
Scott Mc Allister	Research associate	N/A	1.2	Involving animal surgery and collect samples	

7-2. Has there been a change in the activity since the last reporting period? Nothing to report.

7-3. What other organizations were involved with the project as partners? Nothing to report. There was no other organization involved with the project.

8. Special reporting requirement. Nothing to report.
9. Appendices. One 2021 ORS abstract.

A2M Inhibits Chondrocyte Catabolism by Blocking IL-1β/NF-κB Pathway

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INTRODUCTION: Dramatic increases in various inflammatory enzymes have been previously correlated with cartilage degeneration, a hallmark of osteoarthritis (OA). Specifically, IL-1β, a master proinflammatory cytokine, is implicated as an important player in the development of post-traumatic osteoarthritis (PTOA) through upregulation of NF-kB, which activates catabolic enzymes that may mediate the cartilage degradation seen in early PTOA. Alpha 2-macroglobulin (A2M) is major anti-inflammatory cytokine that can inhibit IL-1β and its downstream effects. In recent studies, A2M was shown to reduce catabolism-associated cartilage damage in vitro and in preclinical PTOA models, thus identifying it as a potential therapeutic agent. However, the mechanism of action of A2M in slowing PTOA pathogenesis was unclear. We hypothesized that A2M binds and neutralizes IL-1β, blocking the downstream NF-Kb-induced catabolism seen in PTOA joints. The objective of this study was to test our hypothesis using human (C28) and pig chondrocytes in vitro.

METHODS: Human (cell line C28) and pig chondrocytes were incubated with A2M protein (Sigma)-labeled with VivoTag^{TM} 680 (0.25 mg/ml). Additional chondrocytes were incubated with VivoTag^{TM} 680 alone as a control. All chondrocytes were then treated with IL-1β (8 ng/mL)(Cell signaling) and incubated for 2 days. The location of VivoTag^{TM} 680-A2M post-incubation was detected by confocal fluorescence microscopy. The degree of binding between A2M and IL-1β was evaluated through immunoprecipitation (IP) using the IL-1β antibody (Cell signaling). Catabolic proteins, including IL-1β and NFkB pathway products, were quantified by Western blot.

RESULTS: A2M labeled with VivoTag^{TM} 680 was easily detected in the cytoplasm of C28 human and pig chondrocytes by fluorescence microscopy (Figure 1). IP experiments demonstrated that A2M was able to bind IL-1β. Western blotting revealed that IL-1β upregulated C28 cell NF-κB in a dose-dependent manner (Figure 2A and B), and that A2M dose-dependently reduces IL-1β and NF-κB (Figure 3). These findings indicate that A2M was able to enter chondrocytes and bind IL-1β, which led to reduced NF-κB expression. Thus, A2M was shown to neutralize IL-1β and to reduce the NFkB pathway-linked activation of proinflammatory responses. A2M also inhibited the level of IL-1β in pig chondrocytes, but due to the unavailability of pig-specific antibodies, we were unable to obtain NF-kB data from pig chondrocyte experiments (Figure 2C）.

DISCUSSION: Our findings advance our understanding of the complex anti-inflammatory mechanism of A2M in attenuating the pro-inflammatory responses seen in PTOA development. The results of this study confirm that A2M inhibits the IL-1β /NF-κB signaling pathway in human chondrocytes in vitro, which likely plays a critical role in cartilage degradation, especially in early PTOA. Thus, A2M may be a viable therapy to slow PTOA development in patients with traumatic joint injuries or joint surgeries resulting in significant inflammation. In vivo studies will be needed to confirm this finding.

SIGNIFICANCE/CLINICAL RELEVANCE: A2M inhibits catabolism by binding to IL-1β and blocking the IL-1β/NF-κB signaling pathway. This finding may result in a novel PTOA-preventative therapy utilizing A2M to reduce inflammation-induced catabolic activity and protect cartilage after major joint injuries or surgeries. An example application could be supplemental intra-articular injection of A2M shortly after joint injury, which may in turn prevent the progression of PTOA.

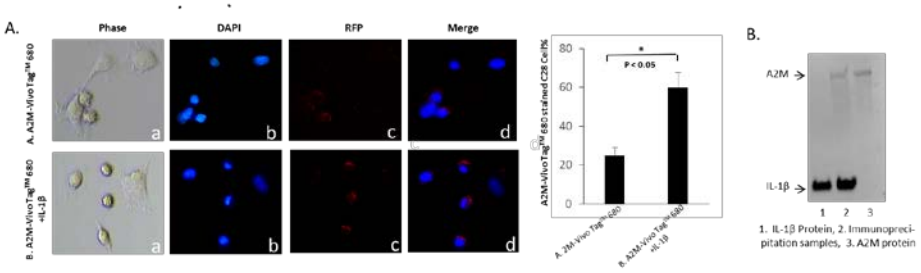


Fig.1. A2M entered into C28 chondrocytes as detected by immunofluorescence assay (A) and binded to IL-1β as detected by IP (B).

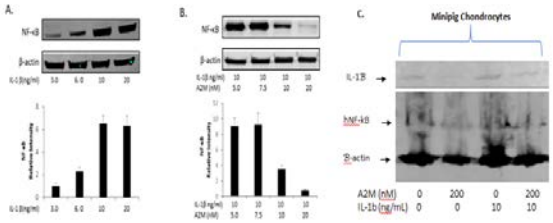


Fig.2. IL-1β induced C28 chondrocyte NF-κB expression in a dose-dependent manner (A). A2M inhibited the level of C28 cell NF-κB induced by IL-1β in a dose-dependent manner as detected by Western blotting (B). A2M inhibited the level of pig chondrocyte IL-1β as detected by Western blotting (C).

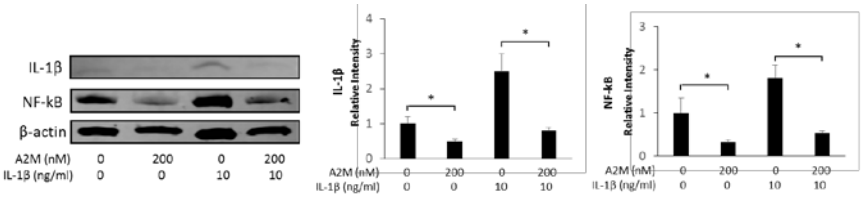
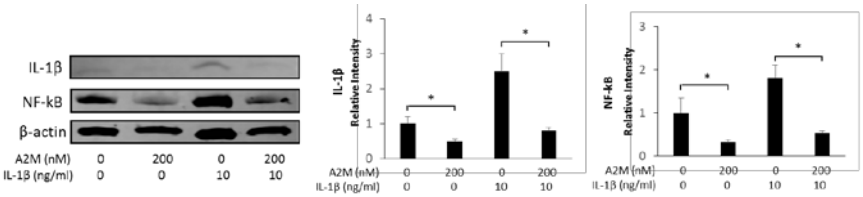


Fig.3. A2M treatment (200nM) reduced both the levels of IL-1β and NF-κB expression in C28 cells detected by Western blotting (left) and the quantitate data of IL-1β and NF-κB was showed (right).



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