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14. ABSTRACT

The purpose of this prospective animal study is to investigate new treatments to improve upper extremity joint function and prevent heterotopic ossification (HO, pathologic bone formation in muscle) follow severe injury. Plasmin is a critical reparative protease, essential for tissue regeneration following injury. The work proposed in this application will delineate the ideal timing for prophylactic plasmin therapy needed for clinical trials in both military and civilian trauma patients at risk for developing HO and associated impaired joint function. If our overarching hypothesis is proven true, the clinical impact is of most importance in the upper extremity as even partial prevention of a shoulder or elbow joint contracture can provide a wounded soldier or civilian with independence in activities of daily living. Importantly, as we have established that plasmin is essential both for preventing HO and promoting fracture repair/bone health, this would be the first therapy that does not compromise bone biology in order to prevent HO.

15. SUBJECT TERMS

Heterotopic Ossification Upper extremity function joint contracture

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1. INTRODUCTION:

It's estimated that over 50% of combat-related injuries sustained by military personnel affect the musculoskeletal system, with musculoskeletal injuries being one of the most common reasons for medical discharge. Heterotopic ossification (HO) is a significant source of morbidity related to these injuries due to loss of joint and muscle function and chronic pain. HO of the elbow and shoulder joints hinders normal joint function and the ability to perform daily activities. Combat-related amputation from injuries to the extremities can also increase the risk of HO development post-surgery. Chronic musculoskeletal conditions not only prevent return to duty, but they can also increase the risk for future injuries. As such, combat-related HO presents a substantial medical burden to the military with long-term consequences.

Treatments for HO include prophylactic drugs, surgical intervention, physical therapy, and radiation therapy, but all of these treatments either lack efficacy or instigate significant adverse effects. Surgical removal of HO is effective, but this is only beneficial if intervention occurs after the HO has matured and it institutes a high risk of hemorrhage and infection, both of which may increase morbidity and medical costs. While prophylactic NSAID therapy remains largely ineffective for the prevention of HO, bisphosphonate therapy, while effective, negatively affects fracture healing and bone remodeling. As such, an effective prophylactic therapy that does not interfere with bone healing or maintenance will not only prevent HO and long-term sequelae but will also circumvent the medical complications associated with the current therapeutic interventions.

The overall objective of this prospective animal study is to investigate new treatments to improve upper extremity joint function and prevent HO following severe injury. Plasmin is a critical reparative protease, essential for tissue regeneration following injury. Findings from our laboratory have demonstrated that in severely injured patients, such as individuals experiencing burn injuries, plasmin is depleted in relation to the severity of injury (as measured by total body surface area burned). Aligning with these clinical results, we observed a marked depletion of plasmin activity in our murine model of thermal injury. Furthermore, when mice received a concomitant burn and tissue injury to their elbow, we observed HO formation and impaired elbow function, akin to results observed in genetically plasminogen deficient animals.

From these results, this proposal is focused on the application of plasmin therapy, as a means to reduce the HO formation and improve upper extremity function following injury. Specifically, the work proposed in this application will delineate the ideal timing for prophylactic plasmin therapy. The proposed research will be conducted using a validated murine skeletal muscle injury at the elbow which results in calcification around the elbow with functional deficits of that upper extremity.

If our overarching hypothesis is proven true, the clinical impact is of most importance in the upper extremity as even partial prevention of a shoulder or elbow joint contracture can provide a wounded soldier or civilian with independence in activities of daily living. Importantly, as we have established that plasmin is essential both for preventing HO and promoting fracture repair/bone health, this would be the first therapy that does not compromise bone biology in order to prevent HO.

The Aims to be examined include:
Aim 1: Determine the therapeutic window of restoring plasminogen consumed by thermal injury with recombinant plasminogen to prevent upper extremity heterotopic ossification and loss of function.

Aim 2: Determine the therapeutic window of enhancing plasmin activity by targeting plasmin's inhibitor (alpha2-anti-plamsin) to prevent upper extremity heterotopic ossification and loss of function.

Aim 3: We postulate that utilizing both methods, within their critical therapeutic windows determined in Aim 1 and 2, will provide the most efficacious treatment that prevents elbow heterotopic ossification and loss of function after a severe thermal injury with a concomitant injury of the elbow.

2. KEYWORDS:

Heterotopic Ossification, Upper extremity function, joint contracture, plasminogen, elbow

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: **Determine the optimal timing of enhancing plasmin activity by targeting its inhibitor α2AP that i) restores plasmin activity, ii) prevents HO and elbow joint contracture.**	**Timeline**	**Site 1**	**Site 2**
Major Task 1: Conduct muscle injury model with/without a2AP ASO administration	**Months**	**Site 1**	**Site 2**
Subtask 1: Obtain ACURO approval	1-3	Dr. Schoenecker	
Subtask 2: Obtain IACUC approval	1-3	Dr. Schoenecker	
Subtask 3: Treatment administered beginning at 0,1,2,3,4,5,6, or 7 days post injury.	3-9	Dr. Schoenecker (80 mice total)	
Subtask 4: Synthesis of fibrin targeting peptide (FTP) for weekly in vivo imaging of fibrin resolution by plasmin	1-6		Dr. McCarthy
Milestone Achieved: Local IACUC and ACURO Approval	3	Dr. Schoenecker	
Milestone: Synthesis of fibrin targeting peptide completed	6		Dr. McCarthy
Major Task 2: Longitudinal Assessment of HO	**Months**	**Site 1**	**Site 2**
Subtask 1: Radiographic Analysis weekly: 7, 14, 21, and 28 DPI	3-9	Dr. Schoenecker	
Subtask 2: MicroCT analysis Endpoint: 28 DPI.	6-12	Dr. Schoenecker	
Subtask 3: Histological Analysis Endpoint: 28 DPI.	6-12	Dr. Schoenecker	
Milestone(s) Achieved: Completed HO assessment with a2AP ASO treatment	12	Dr. Schoenecker	

Major Task 3: Longitudinal Assessment of Elbow Function	**Months**	**Site 1**	**Site 2**
Subtask 1: Grip Strength Analysis weekly: 7, 14, 21, 28 DPI	4-9	Dr. Schoenecker	
Subtask 2: Wire Hang Analysis weekly: 7, 14, 21, and 28 DPI	4-9	Dr. Schoenecker	
Subtask 3: Treadscan Analysis weekly: 7, 14, 21, and 28 DPI	4-9	Dr. Schoenecker	
Milestone Achieved: Completed elbow function assessment with a2AP ASO treatment	9	Dr. Schoenecker	
Major Task 4: Longitudinal Assessment of Plasminogen Levels	**Months**	**Site 1**	**Site 2**
Subtask 1: Serologic Analysis weekly: 7, 14, 21, and 28 DPI	4-12	Dr. Schoenecker	
Subtask 2: Weekly assessment of fibrin resolution by plasmin using in vivo imaging of FTP: 7, 14, 21, and 28 DPI	4-9	Dr. Schoenecker	
Milestone Achieved: Completed assessment of plasminogen levels and plasmin activity with a2AP ASO treatment	12	Dr. Schoenecker	
Major Task 5: Data Analysis	**Months**	**Site 1**	**Site 2**
Subtask 1: Data Analysis	6-12	Dr. Schoenecker	
Milestone Achieved: Completion of all data collection and analysis within Aim 1	12	Dr. Schoenecker	
Specific Aim 2: Determine the optimal timing of restoring plasminogen by administering recombinant plasminogen that i) restores circulating plasminogen levels, ii) prevents heterotopic ossification (HO) and elbow joint contracture	**Timeline**	**Site 1**	**Site 2**
Major Task 1: Conduct muscle injury model with/without recombinant plasminogen administration	Months		
Subtask 1: Treatment administered at 0,1,2,3,4,5,6, or 7 days post injury. 5 mice per group.	12-18	Dr. Schoenecker (80 mice total)	

Subtask 2: Synthesis of FTP	12-18		Dr. McCarthy
Milestone Achieved: Completion of model with recombinant plasminogen administration and FTP synthesis	18	Dr. Schoenecker	
Major Task 2: Longitudinal Assessment of HO	**Months**	**Site 1**	**Site 2**
Subtask 1: Radiographic Analysis weekly: 7, 14, 21, and 28 DPI	12-18	Dr. Schoenecker	
Subtask 2: MicroCT analysis Endpoint at 28 DPI.	15-21	Dr. Schoenecker	
Subtask 3: Histological Analysis Endpoint at 28 DPI.	15-21	Dr. Schoenecker	
Milestone Achieved: Completion of HO assessment following recombinant plasminogen administration	21	Dr. Schoenecker	
Major Task 3: Longitudinal Assessment of Elbow Function	**Months**	**Site 1**	**Site 2**
Subtask 1: Grip Strength Analysis weekly: 7, 14, 21, and 28 DPI	13-18	Dr. Schoenecker	
Subtask 2: Wire Hang Analysis weekly: 7, 14, 21, and 28 DPI	13-18	Dr. Schoenecker	
Subtask 3: Treadscan Analysis weekly: 7, 14, 21, and 28 DPI	13-18	Dr. Schoenecker	
Milestone Achieved: Completed elbow function analysis following recombinant plasminogen administration	18	Dr. Schoenecker	
Major Task 4: Longitudinal Assessment of Plasminogen Levels	**Months**	**Site 1**	**Site 2**
Subtask 1: Serologic Analysis weekly: 7, 14, 21, and 28 DPI	15-21	Dr. Schoenecker	
Subtask 2: Weekly assessment of fibrin resolution by plasmin using in vivo imaging of FTP: 7, 14, 21, and 28 DPI	13-18	Dr. Schoenecker	
Milestone Achieved: Completed assessment of plasminogen levels and plasmin activity with recombinant plasminogen	21	Dr. Schoenecker	

Major Task 5: Data Analysis			
Subtask 1: Data Analysis	16-24	Dr. Schoenecker	
Milestone Achieved: Completion of all data collection and analysis within Aim 2	24	Dr. Schoenecker	Dr. McCarthy
Specific Aim 3: Determine the optimal timing and duration of administration of a2AP ASO + recombinant plasminogen that prevents HO and elbow joint contracture following muscle injury.	**Timeline**	**Site 1**	**Site 2**
Major Task 1: Conduct the muscle injury model with/without a2AP ASO + recombinant plasminogen administration	**Months**	**Site 1**	**Site 2**
Subtask 1: Treatment administered based on optimal dosing points determined in aims 1 & 2. 5 mice per group.	24-30	Dr. Schoenecker (80 mice total)	
Subtask 2: Synthesis of FTP	24-30		Dr. McCarthy
Milestones Achieved: Completion of muscle injury model with combined dosing of therapeutics	30	Dr. Schoenecker	
Major Task 2: Longitudinal Assessment of HO.	**Months**	**Site 1**	**Site 2**
Subtask 1: Radiographic Analysis weekly: 7, 14, 21, and 28 DPI	24-30	Dr. Schoenecker	
Subtask 2: MicroCT analysis Endpoint at 28 DPI.	27-33	Dr. Schoenecker	
Subtask 3: Histological Analysis Endpoint at 28 DPI.	27-33	Dr. Schoenecker	
Milestone Achieved: Completion of HO assessment following combined therapy	33	Dr. Schoenecker	
Major Task 3: Longitudinal Assessment of Elbow Function	**Months**	**Site 1**	**Site 2**
Subtask 1: Grip Strength Analysis weekly: 7, 14, 21, and 28 DPI	24-30	Dr. Schoenecker	
Subtask 2: Wire Hang Analysis weekly: 7, 14, 21, and 28 DPI	24-30	Dr. Schoenecker	

Subtask 3: Treadscan Analysis weekly: 7, 14, 21, and 28 DPI	24-30	Dr. Schoenecker	
Milestone Achieved: Completion of elbow function assessment following combined therapy	30	Dr. Schoenecker	
Major Task 4: Longitudinal Assessment of Plasminogen Levels			
Subtask 1: Serologic Analysis weekly: 7, 14, 21, and 28 DPI	27-33	Dr. Schoenecker	
Subtask 2: Weekly assessment of fibrin resolution by plasmin using in vivo imaging of FTP: 7, 14, 21, and 28 DPI	25-30	Dr. Schoenecker	
Milestone achieved: Completed assessment of plasminogen levels and plasmin activity with both plasminogen and a2AP ASO therapies	33	Dr. Schoenecker	
Major Task 5: Data Analysis and Manuscript Preparation			
Subtask 1: Data Analysis	30-36	Dr. Schoenecker	
Subtask 2: Manuscript Preparation	30-36	Dr. Schoenecker	Dr. McCarthy
Milestones Achieved: Completion of all data collection and analysis within Aim 3 and manuscript preparation of data collected from this proposal	36	Dr. Schoenecker	Dr. McCarthy

What was accomplished under these goals?

Milestone Achieved in Year 1 of Proposal:
Major Task 1, Subtask 1: Obtain ACURO Approval
Major Task 1, Subtask 2: Obtain IACUC Approval
Major Task 1, Subtask 4: Begin synthesis of FTP peptide
Major Task 1, Subtask 3: Begin experiments. Treatment administration a2AP ASO
Major Task 2, Subtask 1: Begin Radiographic analysis weekly.
Major Task 3, Subtask 1-3: Begin Functional Assessments- Grip Strength Analysis/wire hang/tredscan
Major Task 4, Sub task 1-2: Begin assessment of Plasminogen Levels

Milestones to be accomplished in Year 2:
Major Task 1, Subtask 4: Continue synthesis of FTP peptide
Major Task 1, Subtask 3: Continue experiments. Treatment administration a2AP ASO

Major Task 2, Subtask 1: Continue Radiographic analysis weekly.
Major Task 3, Subtask 1-3: Continue Functional Assessments-
Major Task 4, Sub task 1-2: Continue assessment of Plasminogen Levels
Major Task 5, Sub task 1: Begin data analysis
Specific Aim 2- Major Task 1: Obtain recombinant plasminogen from collaborators for use in model.

Milestones accomplished in Year 3:
Major Task 1, Subtask 3: Continue experiments. Treatment administration a2AP ASO
Major Task 2, Subtask 1: Continue Radiographic analysis weekly.
Major Task 3, Subtask 1-3: Continue Functional Assessments-
Major Task 4, Sub task 1-2: Continue assessment of Plasminogen Levels
Major Task 5, Sub task 1: Begin data analysis

Milestones accomplished in Year 4:
Major Task 5, Sub task 1: Continued data analysis
Major Task 1, Subtask 3: Continue experiments. Treatment administration a2AP ASO
Major Task 1, Subtask 3: Continue experiments. Treatment administration of recombinant plasminogen
Major Task 4, Subtask 2: Assessment of fibrin resolution by plasmin using in vivo imaging of FTP: 7, 14, 21, and 28 DPI
Major Task 2, Subtask 1: Continue Radiographic analysis weekly.
Major Task 3, Subtask 1-3: Continue Functional Assessments-
Major Task 4, Sub task 1-2: Continue assessment of Plasminogen Levels
Major Task 5, Subtask 2: Begin Manuscript Preparation

Limitations & Modifications: COVID-19 had a significant impact on Year 2 and Year 3's research goals due to personnel and experimental restrictions. While much has been done to minimize the impact of COVID-19 on this research, a delay in the number of experiments able to be completed was experienced. As a result of this delay, we requested a NCE for year 4. Given that animal protocols expire after 3 years, we necessitated updating our protocols both at the institutional level and with ACURO during this past year. While the institutional protocols were updated on time to begin experiments in the 4th year, ACURO approvals were significantly delayed until 8/31/2021 with our group being notified on 11/8/2021. Once approvals were in place, animals experiment and breeding continued.

The overarching goal of this proposal is to examine therapeutics aimed at i) preventing skeletal muscle calcification and 2) improving elbow function following traumatic injury. Through experiments conducted in year 1, we have demonstrated that a2AP ASO administration at the time of injury effectively reduced the calcification of skeletal muscle surrounding the elbow. Yet, no marked difference in elbow function was observed given that all mice, independent of burn application or therapy, heal well by 28DPI. For this reason, during year 2 we improved our injury model to more effectively examine the therapeutic effect on skeletal muscle calcification and elbow function.

Experimental Findings: Previously, our lab has demonstrated that plasminogen, and its active

form plasmin, is essential for protecting skeletal muscle from calcification and promoting muscle repair (PMID: 27530373). Ongoing clinical work by our laboratory (outside of this grant) has demonstrated that in patients with severe burn injuries, plasminogen antigen levels and plasmin enzyme activity are reduced relative to the severity of the burn injury following a period of hyperfibrinolysis (*JCI-Insight, 2021*). When a proportional amount of plasminogen is depleted in our mouse model (40-50%, Plasminogen (PLG) Heterozygous animals), this leads to marked skeletal muscle calcification following a focal muscle injury, that progresses to HO between 28 and 42 days post-injury. For these reasons, we examined the upper extremity muscle injury in plasminogen heterozygous animals to more effectively model muscle calcification with a persistent diminishment in upper extremity function.

To compare the results in plasminogen deficient (PLG HET) and the burn model, four experimental groups were compared: 1) PLG WT animals + upper extremity injury, 2) PLG WT animals + Burn + upper extremity injury, 3) PLG HET + Upper extremity injury, 4) PLG HET + Burn + Upper extremity injury.

When assessing soft tissue calcification (STiCSS), while no calcification was observed in the PLG WT animals + upper extremity injury (blue), marked calcification was observed in PLG WT mice following burn injury (Group 2, red). As previously observed, the calcification observed in PLG WT animals + Burn + upper extremity injury at 7 days post injury regressed over 42 days post injury resulting in minimal calcification by 42 days post injury.

When assessing PLG HET mice, following injury alone, marked calcification was observed at 7 DPI (Group 3, green). When burn was applied in combination to the plasminogen deficiency, even greater calcification was observed at 7DPI (Group 4, purple). Like WT animals, regression in the amount of calcification was observed over 42D in PLG HET mice +/- burn injury, but the rate was mildly reduced, resulting in persistent calcification within skeletal muscle by 42 DPI.

When assessing upper extremity function, all mice experience comparable drops in grip strength by 7DPI when a focal muscle injury was applied. Importantly, no increased loss of function was observed in the burn injury group. While WT function re-established to over time (group 1 and 2, red and blue), grip strength remained diminished in all PLG HET mice, independent of burn injury (Group 3 and 4, green and purple).

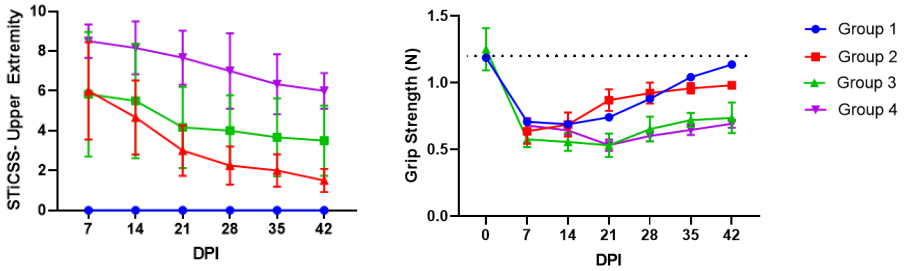


Figure 1: Experimental Model Validation for Soft Tissue Calcification Score (STiCSS) and Grip strength 0-42 days post injury (DPI).

Given these findings, we have worked with the award administrators program directors to shift all future experiments assessing therapeutics aimed at i) preventing skeletal muscle calcification and 2) improving elbow function following traumatic injury to PLG HET mice without burn injury. This change was noted in our updated SOW. While calcification is greater when combined with burn injury, both burned and unburned PLG HET mice develop HO and no additional functional deficient was observed with the addition of burn injury.

Role of Plasminogen in Soft Tissue Calcification and Elbow Function:

The above results clearly demonstrate that LOSS of plasminogen predispose tissues around the elbow to calcification and delayed repair that influences function following injury. Complementary experiments by our laboratory have demonstrated that following injury to a tissue, plasminogen binds actively to sites of injury (**Figure 2A&B**). This corresponds to its function in removing damage matrix, such as fibrin, which is delayed in cases of plasminogen deficiency or burn (**Figure 2C&D**). These experiments align with Specific Aim 1; Major Task 1; Subtask 4, and Specific aim 1; major task 4; Subtask 2.

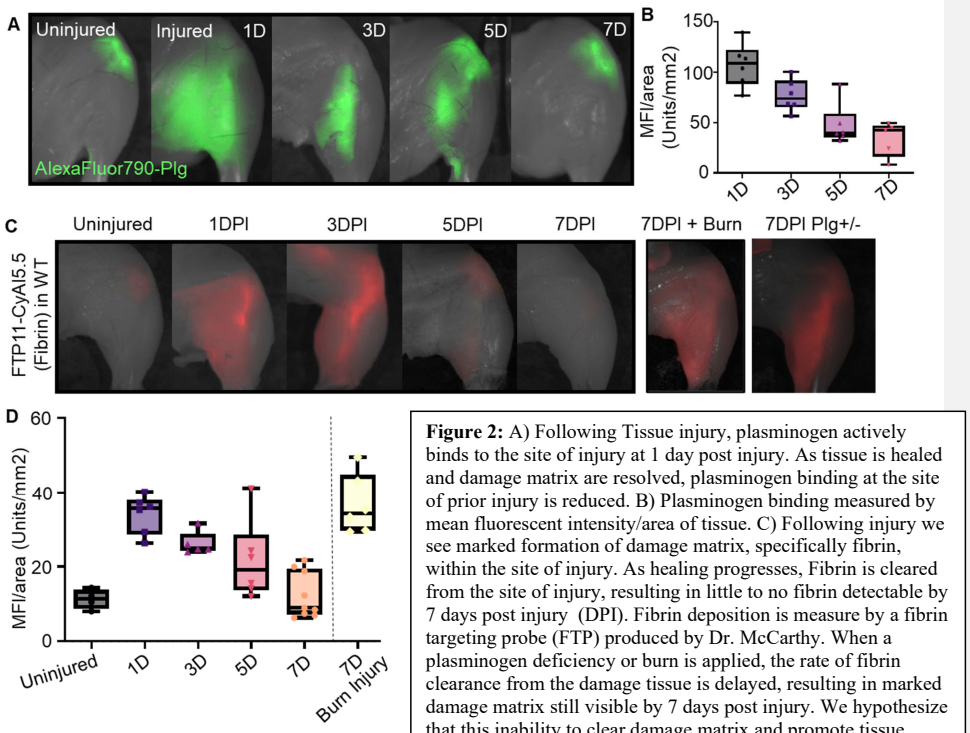


Figure 2: A) Following Tissue injury, plasminogen actively binds to the site of injury at 1 day post injury. As tissue is healed and damage matrix are resolved, plasminogen binding at the site of prior injury is reduced. B) Plasminogen binding measured by mean fluorescent intensity/area of tissue. C) Following injury we see marked formation of damage matrix, specifically fibrin, within the site of injury. As healing progresses, Fibrin is cleared from the site of injury, resulting in little to no fibrin detectable by 7 days post injury (DPI). Fibrin deposition is measure by a fibrin targeting probe (FTP) produced by Dr. McCarthy. When a plasminogen deficiency or burn is applied, the rate of fibrin clearance from the damage tissue is delayed, resulting in marked damage matrix still visible by 7 days post injury. We hypothesize that this inability to clear damage matrix and promote tissue regeneration contribute to changes in tissue function observed around the elbow.

From these observations, we next examined if enhancing plasmin activity following injury reduce the rate of soft tissue calcification and improve elbow function. Aligning with prior reports from our group, we observed that a2AP ASO administered before injury effectively prevents muscle calcification in PLG HETs. Likewise, when recombinant plasminogen was administered to PLG HETs prior to injury, this effectively reduced the amount of calcification formed by 7 days post injury (**Figure 3**). These experiments align with Specific Aim 1; Major task 2 and Specific Aim 2; Major task 2.

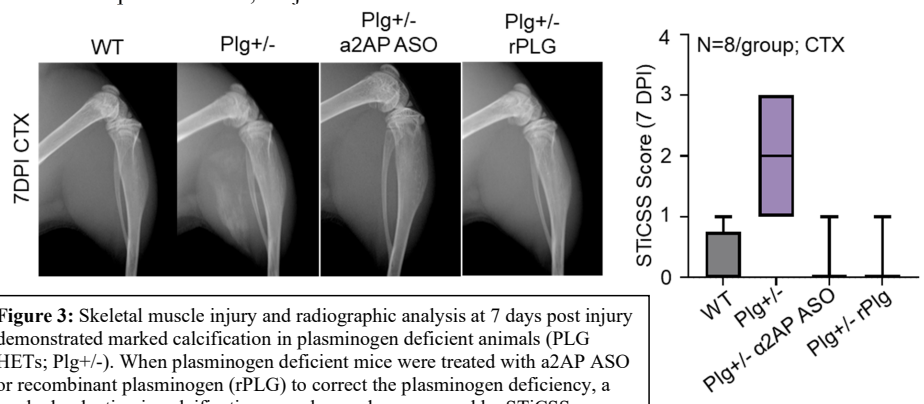


Figure 3: Skeletal muscle injury and radiographic analysis at 7 days post injury demonstrated marked calcification in plasminogen deficient animals (PLG HETs; Plg+/-). When plasminogen deficient mice were treated with a2AP ASO or recombinant plasminogen (rPLG) to correct the plasminogen deficiency, a marked reduction in calcification was observed as measured by STiCSS.

We next examined if these treatment prior to injury would likewise correct the plasminogen deficiency and associated calcification phenotype in the burn model. These experiments directly align with Specific Aim 1 and Specific Aim 2. Here we observed that while a2AP ASO greatly reduced the amount of calcification following injury by 7 days post injury, administration of recombinant plasminogen (rPLG) had little to no effect (**Figure 4**). We hypothesize that this is a result of plasminogen consumption that occurs following the severe burn injury, impacting both the native and recombinant plasminogen. Alternatively, a2AP function by inhibiting plasmin main inhibitor, there by boosting the activity of the remaining plasminogen. Thus, these results indicate that administration of a recombinant protein that is actively been utilized following severe injury may not serve as an effective therapeutic strategy for severely injured patients. Therefore, we chose to not continue with this line of experimentation (Specific Aim 2) or the combination treatment (Specific Aim 3) given the success of a2AP ASO administration (Specific Aim 1). Therefore, we continued our investigation into the effectiveness of a2AP ASO to improve elbow function following injury.

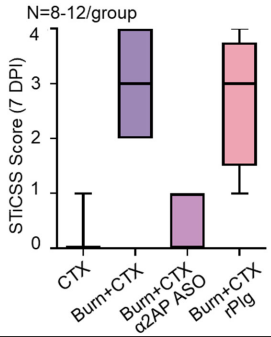


Figure 4: a2AP ASO but not recombinant plasminogen (rPLG) reduces soft tissue calcification at 7 days post injury (7DPI).

When a2AP ASO was administered prior to injury, this resulted in a marked improvement in upper extremity function following injury (Figure 5). These experiments align with Specific Aim 1; major task 3. To account for variability in baseline grip strength between animals, all values

are presented in a % change in grip strength from baseline. Mice were sacrificed at 42 days post injury and tissue samples of the injured upper extremity were collected. We will continue to analyze these samples beyond the funding period with detailed histology to assess for fibrotic changes and inflammatory cellular infiltrate (Specific Aim 1; major task 2; subtask 3).

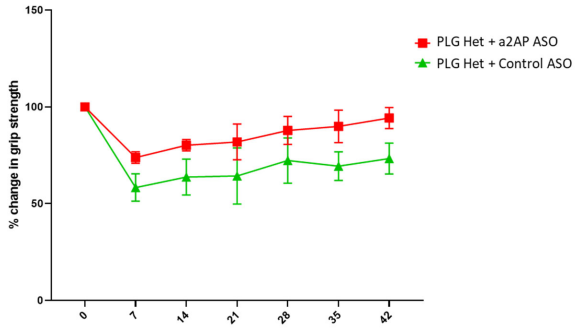


Figure 5: Treatment with a2AP ASO improves elbow function following injury in plasminogen deficient animals.

Interestingly, when applied to WT animals where plasminogen is genetically intact, administration of a2AP ASO likewise had a positive effect on the return to function following an upper extremity elbow injury. This suggests that a2AP ASO has the capacity to allow for supraphysiologic effects of plasmin that may expedite musculoskeletal tissue repair and healing. Future studies beyond this funding period are warranted.

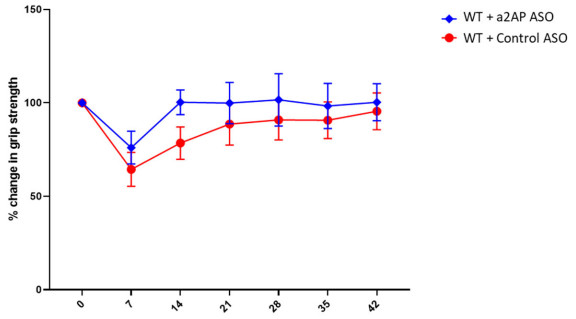


Figure 6: Treatment with a2AP ASO improves elbow function following injury in WT animals.

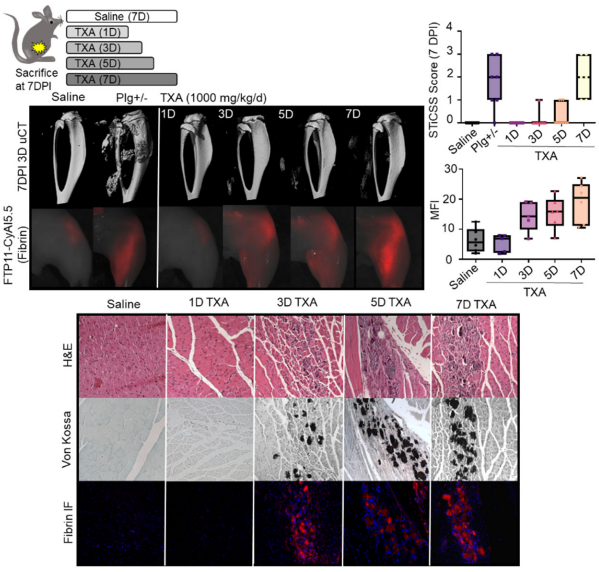
Given these promising results, we will confirm these improvements in elbow function as measured by tredscan analysis and wire hang, though we fully anticipate that results will be recapitulated to those observed in grip strength testing (Major Task 3; Sub task 2-3). Likewise we will continue to assess histologic tissues of treated and un treated samples to observe at a tissue level, what effects a2AP ASO treatment has on tissue regeneration (Major task 2; subtask 3).

Clinical application of these findings:

Given these findings, future studies focused on enhancing plasmin activity at the time of injury are warranted to both limit soft tissue calcification and improve tissue regeneration and function. Contrary to these recommendations, a recent clinical report has demonstrated that pharmacologic administration of TXA, a potent plasmin inhibitor, helped to reduce the prevalence of HO when given at the time of surgery for an elbow trauma. (DOI: 10.2106/JBJS.22.01212). We anticipate that this observation aligns with our and others recent findings that TXA administration has a marked effect on lowering the inflammatory response to injury, which has been suggested in other studies to be a driving factor for HO formation. However, when administered in our burn model, no reduction in the amount of soft tissue calcification was observed (data not shown). Further studies on the mechanism of action are warranted, as well as application of TXA in variable models of traumatic injury at risk for developing HO.

Our group also identified that optimization of the dosing timing of TXA should be investigated. To this point, given the role of plasminogen in preventing calcification and promoting tissue regeneration, we examined if prologued dosing of TXA after an injury would have deleterious effects.

In our murine model of skeletal muscle injury, TXA (1000mg/kg/d) was administered started at the time of injury and continue through 1, 3, 5, or 7 days post injury. All animals were sacrificed at 7 days post injury. Here we observed that with prolonged TXA dosing, more fibrin was remaining within the site of injury by 7 days post injury as measured by FTP. This likewise corresponded with increased soft tissue calcification with prolonged TXA dosing as measured by radiographic analysis (STiCSS) and histology.



These findings demonstrate that the timing and duration of application of TXA should be carefully considered. While clinical data suggests that TXA administration with elbow surgery may reduce risk of HO, this effect could not be reproduced in a small animal model of trauma when administered at the time of injury.

Furthermore, prolonged dosing of TXA as the tissue undergoes regeneration (3-7 days post injury) here was found to have deleterious effects on the removal of the fibrin damage matrix, the amount of soft tissue calcification, and tissue

regeneration. Further studies are necessary to confirm if these changes also impact tissue function.

Major Accomplishments:

- Three manuscripts are currently being produced by the laboratory containing findings from this work. Manuscripts will be submitted in 2023-2024.
- Elements of this work are being prepared for two abstracts to the Orthopaedic Research Society Meeting in 2024.

What opportunities for training and professional development has the project provided?
Zachary Backstrom, who was supported by this award as a research assistant, has been accepted to medical school. This award allowed him to conduct research, learn a great deal about translational research, and learn many scientific skills that helped support his application to medical school.

Dr. Stephanie Moore-Lotridge, who has been supported by this award as a postdoctoral fellow, has been promoted to an assistant professor. This promotion was in part supported by her work on this grant and the mentorship she has provided others as part of this award.

How were the results disseminated to communities of interest?
Three manuscript are currently being produced by the laboratory containing findings from this work.

What do you plan to do during the next reporting period to accomplish the goals?
This is the final reporting period. Analysis of the gathered data will continue into the post funding period.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
This work has examined multiple therapeutic aimed at altering plasmin activity and their effect on soft tissue calcification, tissue regeneration, and function. These findings will help guide clinical trials aimed at improving outcomes following injury, help identify optimal dosing strategies, and which therapeutic are best applied in variable clinical scenarios.

What was the impact on other disciplines?
These findings will impact orthopaedic care, trauma care, and all medical specialties involved with rehabilitation and recovery from injury.

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Nothing to report in this period.

Actual or anticipated problems or delays and actions or plans to resolve them
As a result of experimental delays due to COVID-19 restrictions, we were approved for a 4th year NCE. As part of this extension, we necessitated updating our protocols both at the institutional level and with ACURO. While the institutional protocols were updated on time to begin experiments in the 4th year, ACURO approval has been significantly delayed. We were notified on 11/8/2021 that we have received ACURO and moved ahead with breeding and studies at this point.

Changes that had a significant impact on expenditures
COVID-19 has had a significant impact on years 2 and 3's research goals due to personnel and experimental restrictions. While much has been done to minimize the impact of COVID-19 on this research, we did experience a delay in result production. As noted above, we have applied for a NCE for a 4th year to complete studies

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report

Significant changes in use or care of human subjects
Not Applicable

Significant changes in use or care of vertebrate animals.
Due to protocols expiring, institutional and ACURO protocols necessitated updating and re-approval. No significant changes were made to the protocols, but we have been significantly delayed due to not receiving ACURO approval.

Significant changes in use of biohazards and/or select agents
Not Applicable

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Nothing to Report
  Journal publications. Nothing to Report
  Books or other non-periodical, one-time publications. Nothing to Report
  Other publications, conference papers, and presentations. Nothing to Report
- **Website(s) or other Internet site(s)**
  Nothing to report
- **Technologies or techniques**
  Nothing to report
- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

  Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Commented [SM1]: Glenna to update

Name: *Jonathan G Schoenecker*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): 0000-0002-3097-5496
Nearest person month worked: *2*
Contribution to Project: *Dr. Schoenecker oversaw the IACUC and ACURO submission process and directed the planning of upcoming experiments. Dr. Schoenecker worked with Dr. Moore-Lotridge to analyze all data obtained from this proposal period.*

Name: *Stephanie Moore-Lotridge*
Project Role: *Post-doctoral Fellow*
Researcher Identifier (e.g. ORCID ID): 0000-0002-3045-4199
Nearest person month worked: *3*
Contribution to Project: *Dr. Moore-Lotridge conducted the proposed experiments to produce the reported data on the efficacy of a2AP ASO administration at the time of injury for preventing muscle calcification.*

Name: *John C. Reese*
Project Role: *Research Assistant*
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: *1*
Contribution to Project: Mr. Reese is responsible for mouse colony maintenance, laboratory management, and ordering of products needed for experiments.

Name: *Zachary Backstrom*
Project Role: *Research Assistant*
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: *2*
Contribution to Project: Mr. Reese is responsible for mouse colony maintenance, laboratory management, and ordering of products needed for experiments.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Organization Name: Masonic Medical Research Institute (Dr. Jason McCarthy)
Location of Organization: Utica, NY

<u>Partner's contribution to the project</u>: Production of fibrin imaging agents for use in our animal models

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: Submitted