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TITLE:
Mitochondrial Based Treatments that Prevent Post-Traumatic Osteoarthritis in a Translational Large Animal Intraarticular Fracture Survival Model

PRINCIPAL INVESTIGATOR:
Todd O. McKinley, M.D.
James A. Martin, Ph.D.

CONTRACTING ORGANIZATION:
University of Iowa

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The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
The purpose of this research is to investigate a novel therapeutic approach to preventing PTOA by addressing mitochondrial dysfunction and oxidative damage in cartilage. Thus far we have tested a number of compounds for therapeutic activity in a chondral injury model that involves a high energy impact to the medial femoral condyle of rabbits. The selection of compounds included an oxidant scavenger, (N-Acetylcysteine), a drug that reduces mitochondrial superoxide production by blocking electron flow through complex I (amobarbital), and two drugs that block actin and tubulin remodeling (cytochalasin B and nocodazole respectively). Treatments were administered acutely after injury (N-acetyl cysteine and amobarbital) or prior to injury (cytochalasin B and nocodazole). At seven days post-operatively the rabbits were euthanized and the injured cartilage was analyzed for viability, and ATP. While NAC at a low dose was effective in preventing injury-related chondrocyte losses, amobarbital, nocodazole, and cytochalasin B were more effective at sparing metabolic activity. With these data in hand we are ready to go forward with the long-term rabbit study, in which we will determine the effects of amobarbital and nocodazole on cartilage degeneration (Aim 2). The most effective treatment will be tested in a porcine intraarticular fracture model (Aim 3).
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Introduction

Experimental Overview

The purpose of this research is to investigate a novel therapeutic approach to prevent PTOA by treating mitochondrial dysfunction in chondrocytes resulting from an intraarticular injury. We have shown that scavenging excessive injury-related mitochondrial oxidants, or preventing their excessive formation after a severe impact injury to cartilage in a tissue-level model prevented chondrocyte death in a bovine explanted tibial cartilage \(^1-^3\). Subsequently, we demonstrated that oxidant production is strain–dependent and that physiologic levels of mitochondrial oxidants, generated in tissue samples subjected to normal loads, were important promoters of chondrocyte glycolytic ATP synthesis\(^4,^5\). These findings are the basis of the treatment strategies pursued in this in vivo investigation.

Summary of Progress

Thus far we have tested a number of compounds for therapeutic activity in a rabbit model of chondral injury that involves a high energy impact to the medial femoral condyle. The selection of compounds included an oxidant scavenger, (N-Acetylcysteine), a drug that reduces mitochondrial superoxide production by blocking electron flow through complex I (amobarbital), and two drugs that block actin and tubulin remodeling (cytochalasin B and nocodazole respectively). Treatments were administered acutely after surgery (N-acetyl cysteine and amobarbital) or 4 hours prior to surgery (cytochalasin B and nocodazole). At seven days post-op the rabbits were euthanized and the injured cartilage was analyzed for viability, proline incorporation, and ATP and glycosaminoglycan content. Although data analysis is not quite complete, it is fairly clear at this stage that while NAC at a low dose was effective in preventing injury-related chondrocyte losses, amobarbital, nocodazole, and cytochalasin B were more effective at sparing metabolic activity. None of the drugs had significant effects on proline incorporation or glycosaminoglycan content. With these data in hand we are ready to go forward with the long-term rabbit study, in which we will determine the effects of amobarbital and nocodazole on cartilage degeneration (Specific Aim 2).

Body

The three Specific Aims, the associated Statement of Work, and progress to date are outlined below:

Specific Aim 1. Measure changes in chondrocyte ATP production, oxidant production, biosynthetic activity, viability, and PTOA in a survival rabbit model of cartilage injury treated with oxidant scavenging or electron transport complex I inhibition. Subsequently, determine the therapeutic effect of adjuvant glycolytic enhancement.

Task 1: Surgical injury to the rabbit medial femoral condyle (months 1-18)

1. Experimental Groups (sacrifice at either 7 days, 42 days, or 6 months)
   a. Amobarbital
   b. NAC
   c. NAD
   d. Amobarbital with NAD
   e. NAC with NAD

2. Control groups
   a. Impact controls (injury with no treatment)
   b. Sham control (surgery no injury)
   c. Normal
**Task 2:** Confocal, Biochemical, and Histologic Analysis (months 1-21)

1. Confocal Imaging (live cell imaging; oxidant production)
2. Biochemical Analysis (ATP content; proline incorporation; proteoglycan content)
3. Histologic Analysis (Safranin O for Mankin scores; immunohistology for MMP3 and MMP13)

**Specific Aim 2:** Measure changes in chondrocyte ATP production, chondrocyte ROS production, chondrocyte biosynthetic activity, chondrocyte viability, and PTOA in a survival rabbit model of cartilage injury treated with compounds that dissolve filamentous actin and tubulin. Subsequently determine the therapeutic effects of adjuvant glycolysis-enhancing substrate with cytoskeletal dissolution agents.

**Task 1:** Surgical injury to the rabbit medial femoral condyle (months 1-18)

1. Experimental Groups
   a. Cytochalasin B
   b. Nocodazole
   c. Cytochalasin B with NAD
   d. Nocodazole with NAD

2. Control Groups (same as Specific Aim 1)

**Task 2:** Same as Specific Aim 1

**Work to Date Specific Aims 1 and 2**

A rabbit chondral injury model was developed and validated for this project. The stifle joint is rigidly fixed in extension and a posterior arthrotomy is performed to access the weight-bearing portion of the medial condyle. A pendulum device imparts a blow to a 3 mm metal indentor resting on the cartilage surface. The device is instrumented with an accelerometer that records the impaction event at 100 x 10^3 Hz. The accelerometer’s force/time readout reveals the duration of the impact event (typically a few milliseconds) and peak force imparted to the condyle (Figure 1). For treatment testing purposes we chose the 2 J energy level, which generates peak forces of 800 N (113 MPa) and loading rates in the gigapascal/second range. This leaves a partial-thickness cartilage injury, whereas higher energies tend to dislodge cartilage from subchondral bone (Figure 2).

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**Figure 1.** Effects of increasing impact energy on peak impact force and cartilage defect size. Impact energy was related to peak force measured by an accelerometer (A) Cartilage defect size expressed as a percentage of the whole histology section depended on energy and peak force.
Figure 2. Partial-thickness lesion at a 2J impact site on the rabbit medial femoral condyle. Both panels show the same safranin-O/fast green-stained sagittal section through an impact site located on the posterior medial femoral condyle (arrows). The joint was harvested at one week post-op. Local structural damage and GAG depletion can be seen in the low magnification image on the left (bar = 2 mm) and higher magnification image on the right (bar = 300 microns).

Initially we sacrificed rabbits at either 3 days or 7 days post-op. These preliminary experiments dictated that for short term experiments, sacrifice at 7 days would best show us any treatment effects.

After euthanasia the distal femur was stained with calcein AM and injury sites were scanned for viable cell density (Figure 3). The distal femurs were then incubated overnight in media containing $^3$H-proline. For biochemical analyses, cartilage from the entire medial condyle (20-30 mg) was harvested, weighed, and digested with papain for $^3$H-proline incorporation, GAG, and ATP assays. Although we planned originally to perform histologic analyses to document cartilage damage and MMP expression we found that all of the cartilage was required for the biochemical measures, which were more pertinent to detecting treatment effects. However, histology will still be the primary measure employed in Aim 2 to measure cartilage degeneration at 8 weeks post-op.

Figure 3. Viability at an impact site one week after injury. A distal femur was stained with calcein AM and imaged with a confocal microscope to identify live cells (green) cells. The darkened area in the middle of the image indicates loss of viable chondrocytes at the impact site.
Some of the outcome measures we originally proposed proved to be uninformative and have been abandoned. Attempts to measure oxidant production using confocal imaging of dihydroethidium staining failed to reveal a consistent injury-related increase in oxidant levels. This finding was likely due to the 7 day post-op delay, which gave time for the acute burst of impact-induced oxidant production to subside. ³H-Proline incorporation increased with injury, but there were no treatment effects on this response. Since ³H-proline incorporation assays are particularly expensive and labor intensive, we chose to eliminate them from the protocol. In addition, we dropped glycosaminoglycan (GAG) assays from the menu of outcome measures, as GAG cartilage content was unaffected by injury or treatment. This was not surprising given that GAG depletion at this early stage was confined to impact sites and had not spread into the surrounding uninjured cartilage.

Testing of NAD in preliminary in vitro experiments in our explant model failed to show an effect on metabolic responses. Thus, we focused our efforts on the treatments listed in the Table above. Viability and ATP assays both showed significant injury and treatment effects (Figure 4). Compared to uninjured cartilage, both parameters were sharply reduced in untreated injured cartilage. All of the treatments except cytochalasin B had significant protective effects on viability.

**Figure 4.** Effects of treatment on chondrocyte viability and ATP content in injured cartilage. Injured cartilage was imaged by confocal microscopy to determine viable cell density (A), and then assayed for ATP content (B). Cartilage from contralateral limbs (CL) was used as uninjured controls. Injured joints were untreated (No Rx), or treated NAC at 2, 5, or 20 mM (NAC2, NAC5, NAC 20), Cytochalasin B (CytoB), nocodazole (Nocod), or amobarbital (Amobarb). Columns and error bars indicate means and standard errors. The numbers above the columns show p values for treated versus untreated (One-way ANOVA).

<table>
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<th>Dose Groups</th>
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<tr>
<td>1. No Rx</td>
<td>9</td>
</tr>
<tr>
<td>2. 2mM N-acetylcysteine</td>
<td>5</td>
</tr>
<tr>
<td>3. 5mM NAC</td>
<td>5</td>
</tr>
<tr>
<td>4. 20mM NAC</td>
<td>5</td>
</tr>
<tr>
<td>5. 20µM Cytochalasin B</td>
<td>5</td>
</tr>
<tr>
<td>6. 10µM Nocodazole</td>
<td>4</td>
</tr>
<tr>
<td>7. 2.5mM Amobarbital</td>
<td>5</td>
</tr>
</tbody>
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Table. Dose groups for the short-term rabbit study
NAC at 2 mM and 5 mM and nocodazole at 10 µM showed the strongest effects (p < 0.001), followed by 2.5 mM amobarbital (p=0.001) and 20 mM NAC (p=0.005). Viability was increased by cytochalasin B treatment, but this effect just missed statistical significance (p = 0.065). The injury effect on ATP was profound and was not reversed by NAC at any of the three doses tested. On the other hand, cytochalasin B and amobarbital at concentrations used in explant experiments showed promising effects. Both drugs improved metabolism in injured cartilage: While the ATP content of injured cartilage in untreated animals was only 38% of that measured in uninjured cartilage, levels in cytochalasin B- and amobarbital-treated cartilage were 69% and 75% of uninjured respectively.

**Specific Aim 3:** Determine the efficacy of treatments that prevent ROS overproduction, scavenge ROS, or dissolve the cytoskeleton in mitochondria on preventing PTOA in a large animal IAF survival model.

**Task 1:** Surgical creation of a physiologic realistic intraarticular fracture in the Yucatan minipig. Once treatments are optimized in the small animal model, we plan on investigating the optimized treatments in the minipig model.

1. Experimental Groups (months 24 – 40): we plan on investigating four different treatment combinations.
2. Control Groups (months 1 – 15)
   a. Injured controls
   b. Uninjured Controls

**Work to Date on Specific Aim 3**

Although treatment testing in swine will not be initiated until after the long term study in rabbits is completed we have already perfected the fracture and fixation technique. A total of 38 minipigs have had an intraarticular fracture of their hock joint and have been surgically stabilized in either an anatomic position with rigid fixation, an anatomic position with semi-rigid fixation, or in a malreduced position. This work has been partially funded with this grant, and is also being funded by another DoD Grant (Jessica Goetz Principal Investigator). A manuscript documenting the model has been published.

**Related Work**

Our NIH CORT Grant funds a Joint Trauma Biomarker Core dedicated to identifying prognostic molecular markers for PTOA. Synovial and serum fluids collected from porcine and lapine models used in our DoD projects offer unparalleled opportunities for biomarker discovery, which we recently took advantage of by analyzing joint fluid samples from fractured porcine joints for markers associated with alarmin signaling and joint inflammation. In previous DoD-funded research we discovered that cells known as chondrogenic progenitor cells (CPCs) respond to cartilage injury by overexpressing multiple proinflammatory cytokines and chemokines including the potent monocyte chemoattractant CxC motif ligand 12 (CXCL12). We showed that the CPC response was to high mobility group box 1 (HMGB1), an alarmin released from chondrocytes killed by impact injury. As a follow up to these explant studies we sought to determine if CXCL12 and HMGB1 are elevated in...
fractured porcine hock joints. These immuno assays revealed that both peptides were present at higher concentrations in fractured joints than in contralateral uninjured joints at 1 and 2 weeks post-injury (Figure 5). These results are consistent with our hypothesis that CPCs promote acute synovitis via alarmin signaling. We are currently using the hock fracture model to test treatments designed to block HMGB1 and CXCL12.

**Figure 5.** Fracture-related increases in the pro-inflammatory peptides HMGB1 and CXCL12 in joint fluids. Joint fluids were harvested from injured left hock joints (L) and uninjured right hock joints (R) 3 days after fracture (D3), and 1, 2, 4, and 12 weeks after fracture (W1, 2, 4, 12). Enzyme linked immunosorbent assays (ELISA) were used to measure HMGB1 and CXCL12 in the fluid samples. P values are given for t-tests comparing L versus R at each time point (red type). The columns and error bars indicate means and standard deviations based on 4-9 animals per group.

**Key Research Accomplishments**

1. The short-term rabbit studies have concluded and drugs that will be tested in long term studies have been identified (Amobarbital, nocodazole).
2. Significant progress has been achieved in implementing the porcine hock fracture model. The model is producing PTOA in a highly reproducible manner with consistent coronal fractures of the anterior distal tibia.
3. We are beginning to exploit the models to test the hypothesis that alarmin signaling starts the inflammatory cascade that leads to synovitis in injured joints.
Reportable Outcomes.


Conclusions

1. Drugs that block the mitochondrial mechanotransduction pathway that leads to superoxide release preserve cartilage viability and metabolic activity after injury.
2. Oxidant scavenging after an intraarticular impact injury saves cells but reduces metabolic activity.
3. Intraarticular fracture results in elevation of pro-inflammatory peptides that are related to CPC injury responses.

References


