Validation of a Low-Cost Portable Device for Inducing Noninvasive Anterior Cruciate Ligament Injury in Mice

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Abstract

Introduction

Osteoarthritis (OA) is the most common degenerative joint disease and is the leading cause of disability in the United States [1], currently affecting over 32 million US adults [2]. Specifically, OA of the knee is the most prevalent, affecting over 15 million people in the US [3]. Individuals who suffer previous trauma to the knee joint such as anterior cruciate ligament (ACL) rupture are nearly 4 times more likely to develop OA of the knee than uninjured people [4]. About half of individuals diagnosed with ACL rupture or meniscus tear develop post-traumatic osteoarthritis (PTOA) within 10-20 years [5].

Animal models of OA are essential tools for studying the etiology of the disease and potential therapies for slowing or preventing OA progression. Mouse models in particular are critically important due to the abundance of genetic models and the compressed and predictable timeline of disease progression. The recent development of non-invasive mouse models of PTOA is an important step for mechanistic studies of OA, since these models initiate OA with externally applied mechanical loads [6], closely resembling the initiation of human PTOA since no surgical procedures or intra-articular injections are used. Our lab has published several studies using tibial compression overload to cause ACL rupture (ACL-R) [7-14]. This is a simple and quick method that produces a consistent and reproducible joint injury that initiates a rapidly progressing OA in the mouse knee joint. However, this injury method typically requires access to an electromagnetic materials testing system (Electroforce 3200 or comparable). These systems may be prohibitively expensive for some investigators. Additionally, these systems system are physically large and heavy, so they cannot easily be brought into vivaria or barrier facilities.

These limitations make the ACL-R model unfeasible for some studies of PTOA etiology or treatment.

To address these limitations, a group of Mechanical Engineering students at Montana State University designed a low-cost and portable **C**ustom-made **A**CL **R**upture **D**evice (CARD) for initiating joint injuries in mice. This system weighs ~8 kg allowing for more portability, and is a fraction of the cost of the materials testing systems typically used for this application. In this study we validated the CARD system relative to a standard system for ACL-R of mice, including assessment of post-injury joint laxity, subchondral bone changes, osteophyte formation, synovitis, and PTOA progression. Successful validation of this device would make ACL-R in mice more financially accessible and more feasible for some studies due to the portability of the system.

Methods:

Design of the Custom-Made ACL Rupture Device (CARD)

Design requirements for the CARD system included sterilization, force, velocity, stopping ability, data acquisition, and cost. The apparatus was designed to be portable and user friendly, sterilizable with ethanol or other cleaning agents, with force and displacement limits of 14 N and 2.25 mm, respectively. The system was designed to apply a displacement rate of 200 mm/s, which we have previously shown to cause a mid-substance ACL rupture without an avulsion [8]. Finally, the apparatus was designed to cost less than $5,000 (at the time of design). Images and technical drawings of the CARD system are shown in **Fig. 1**. For a fully detailed system

description and files/supplies list for constructing the CARD system, please see the Supplementary Materials.

Animals:

A total of 30 male C57BL/6J mice, 12 weeks old at the time of injury, were obtained from Jackson Laboratory (Bar Harbor, ME). Half of the mice (n=15) were randomly assigned to undergo ACL-R using the current gold standard loading system (ElectroForce 3200, TA Instruments, New Castle, DE), and the remaining mice (n=15) were assigned to undergo ACL-R using the CARD system. Each experimental group was divided into three time points: euthanasia immediately following injury (n=5 mice/group), or 2 weeks (n=5) or 6 weeks (n=5) after injury. Knee joints from mice that were euthanized immediately after injury underwent anterior-posterior (AP) joint laxity as described below. Knee joints from mice that were euthanized 2 or 6 weeks after injury were analyzed with micro-computed tomography to evaluate epiphyseal trabecular bone and osteophyte formation, and were analyzed with whole-joint histology to measure synovitis and PTOA progression.

Non-Invasive ACL Rupture by Tibial Compressive Overload

Tibial compression overload was performed using the ElectroForce 3200 (ELF) as previously described [8]. Briefly, mice were anesthetized with isoflurane inhalation, then the right lower leg was positioned between an upper platen that held the flexed ankle at approximately 30 degrees of dorsiflexion and a lower platen that held the flexed knee. A preload of 1 N was applied to the knee before a single compressive load was applied to a target displacement of -1.7 mm at a loading rate of 200 mm/s.

Tibial compression overload using the CARD system was performed using similar methods. The mouse was anesthetized using isoflurane inhalation, and was then loaded in a prone position with the right knee in the knee cup. The Voice Coil Actuator (VCA) with the ankle holster was then manually lowered to the level of the mouse's foot to stabilize the ankle, leg, and knee joint. Once the mouse leg was stabilized and the VCA was locked in place, the program was initiated and the VCA slowly adjusted the ankle holster until a 2 N preload was applied to the leg. Once a preload of 2 N was reached, the VCA compressed the lower leg at a speed of 200 mm/s. The VCA compressed the lower leg until one of three conditions is met: 1) the force load detected by the load cell drops indicating an ACL rupture, 2) the 14 N maximum load is met, or 3) a displacement limit of 2.25 mm is met. The latter two endpoints ensure that tibial compression is stopped before tibial fracture or damage to other joint structures occurs. After injury, all mice were given a subcutaneous injection of buprenorphine (0.5 mg/kg body weight) for analgesia. Mice were allowed normal cage activity until euthanasia.

Assessment of Anterior-Posterior Joint Laxity

AP joint laxity was analyzed as previously described [8, 15] for both the injured and contralateral uninjured knees immediately following non-invasive ACL-R for both ELF-injured mice (n=5) and CARD-injured mice (n=5). Briefly, the distal tibia and proximal femur were fixed in brass tubes with polymethyl methacrylate (PMMA) and knees were tested at 60˚of flexion with five loading cycles to a target force of ±1.5 N at a rate of 0.5 mm/s. The force was applied normal to the longitudinal axis of the tibia, and the tibia was allowed to translate and rotate about its

longitudinal axis during loading. Total AP joint laxity was computed based on the difference between displacement at +0.8 N and -0.8 N.

Micro-Computed Tomography Analysis of Epiphyseal Trabecular Bone

Knee joints from mice euthanized 2 and 6 weeks post-injury (n=5 mice/time point/experimental group) were scanned using micro-computed tomography (SCANCO μCT 35, Brüttisellen, Switzerland) to analyze epiphyseal trabecular bone microstructure. Scans were performed according to rodent bone structure analysis guidelines (X-ray tube potential = 55 kVp, intensity = 114 mA, 10 μm isotropic nominal voxel size, integration time = 900 ms) [16]. The trabecular bone volume of interest included all trabecular bone distal to the epiphyseal growth plate of the distal femur. Trabecular bone volume fraction (BV/TV), trabecular thickness (Tb.Th), and other microstructural outcomes were measured using the manufacturer's analysis software.

Micro-Computed Tomography Analysis of Osteophyte Volume

Osteophyte analysis was performed on knee joints from 6 week post-injury mice as previously described [11]. Briefly, contours were drawn to include the patella, fabellae, meniscal mineralized tissue, and non-native bone growth on and around the distal femur and proximal tibia for both knee joints. The difference between bone volume of the injured knee vs. the uninjured contralateral knee was calculated to quantify osteophyte volume in the injured knee.

Histological Assessment of Synovitis and OA Progression

Whole-joint histology was used to evaluate overall joint degeneration (OA scoring) and synovitis at both the 2-week time point (n=5 mice/group) and 6-week time point (n=5 mice/group). After

euthanasia, knee joints were fixed in 4% paraformaldehyde for 5-7 days, and were then decalcified using 0.5M EDTA and processed for standard paraffin embedding. Sagittal sections were cut across the whole joint separated by 100 μm, and were stained with toluidine blue. Three graders independently graded OA and synovitis for both the medial and lateral compartments, and scores were averaged across all three graders. Synovitis was scored using a 6-point system consisting of the sum of a synovial lining score on a scale of 0-3 and a synovial stroma cellularity score on a scale of 0-3 as described by Krenn et al. [17]. OA scoring for joint degeneration was scored on a scale of 0-10 with an emphasis on articular cartilage degeneration and/or tibial degeneration. A score of 0-4 indicates the absence of gross tibial degeneration with 0 being a healthy joint and a 4 being a joint with mostly intact articular cartilage that lacks toluidine blue staining. A score of 5-10 indicates a joint with tibial cartilage degeneration, with a 5 being a joint with some tibial degeneration but with more than 90% stained cartilage on the femoral condyle, and 10 being a joint with extensive tibial degeneration and less than 10% stained cartilage on the femoral condyle.

Statistical Analysis:

All data were analyzed at each time point using paired t-tests to compare right vs. left limbs of mice (joint laxity test and μCT analysis) and unpaired t-tests to compare ELF vs. CARD groups (all outcomes) using Prism 9 software (GraphPad by Dotmatics, Boston, MA). All data are presented as mean ± standard deviation. Significance was defined *a priori* as $p<0.05$ for all tests.

Results

Non-Invasive ACL Rupture by Tibial Compressive Overload

Successful ACL-R was confirmed in mouse knees using μCT imaging and whole-joint histology. Of the 15 mice loaded with the CARD system, 13/15 were successfully injured. The ACL was intact for the remaining 2 mice (both from the week 6 group), so they were removed from further analyses. All of the mice loaded with the ELF system were successfully injured.

Assessment of Anterior-Posterior Joint Laxity

As expected, ACL-R resulted in notable increases in AP joint laxity for mice loaded with either the CARD or ELF systems (**Fig. 2**). Mice loaded with the CARD system had AP joint laxity of 1.74±0.24 mm in the injured leg compared to 0.82±0.15 mm in the uninjured contralateral leg (+112%). Mice loaded with the ELF system had AP joint laxity of 1.61±0.10 mm in the injured leg compared to 0.93±0.13 mm in the uninjured contralateral leg (+72%). No significant differences were observed between the CARD and ELF systems.

Micro-Computed Tomography Analysis of Epiphyseal Trabecular Bone and Osteophyte Volume

Non-invasive ACL injury resulted in a loss of epiphyseal trabecular bone in the injured joint of mice injured with both the ELF system and the CARD system at both week 2 and week 6 post-injury (**Fig. 3**). At week 2 post-injury, injured knees from mice loaded with the ELF system had an 11% decrease in BV/TV and a 9% decrease in Tb.Th compared to the uninjured contralateral leg, while injured knees from mice loaded with the CARD system had an 20% decrease in BV/TV and a 15% decrease in Tb.Th compared to the uninjured contralateral leg. At week 6 post-injury, injured knees from mice loaded with the ELF system had an 18% decrease in BV/TV and an 11% decrease in Tb.Th compared to the uninjured contralateral leg, while injured knees from mice loaded with the CARD system had an 18% decrease in BV/TV and a 9%

decrease in Tb.Th compared to the uninjured contralateral leg. No significant differences were observed between ELF and CARD experimental groups for any μCT outcomes.

As expected, ACL-R resulted in considerable formation of osteophytes by week 6 post-injury (**Fig. 3**). Injured knees from mice loaded with the ELF system had mineralized osteophyte volumes of 1.86±0.76 mm^3, while injured knees from mice loaded with the CARD system had mineralized osteophyte volumes of 1.61±0.07 mm^3. No significant differences were observed between experimental groups.

Histological Assessment of OA Progression

Non-invasive ACL injury led to OA progression in the injured joints of mice loaded with both the ELF and CARD systems at 2 and 6 weeks post-injury (**Fig. 4**). At 2 weeks, OA scores for mice injured with the CARD system were increased compared to those injured with the ELF system. The medial and lateral OA scores for mice injured by the CARD system were 4.8±1.7 and 3.1±0.6, respectively, while the medial and lateral OA scores for the mice injured by the ELF system were 2.6±2.7 and 1.3±0.5, respectively. At 6 weeks post-injury, the OA scores for the injured joints of mice loaded with the CARD system didn't exhibit a large amount of progression relative to week 2, while the injured joints of mice loaded with the ELF system exhibited greater disease progression during this time, resulting in similar scores between groups at week 6. The medial and lateral OA scores for mice injured with the CARD system were 7.2±0.6 and 3.7±1.2, respectively, while the medial and lateral scores for mice injured with the ELF system were 6.8±0.4 and 4.8±2.6.

Histological Assessment of Synovitis

Non-invasive ACL injury also led to synovitis in the injured joints of mice loaded with both the ELF and CARD systems at 2 and 6 weeks post-injury (**Fig. 5**). Medial and lateral synovitis scores at 2 weeks post-injury of joints loaded with the CARD system were 3.7±1.1 and 2.9±0.5, respectively, while the ELF system counterparts were 2.9±0.9 and 1.9±0.7, respectively, for the medial and lateral aspects of the joint. At 6 weeks post-injury, medial and lateral synovitis scores of joints loaded with the CARD system were 3.2±0.6 and 3.2±0.6, respectively, while the ELF system counterparts were 3.9±0.4 and 3.0±0.8, respectively.

Discussion

In this study we validated a low-cost portable system for inducing ACL-R in mice. We found that this system was able to induce a joint injury and rate of PTOA in mice that is comparable to a conventional materials testing system (ElectroForce 3200). This novel CARD system may make the ACL-R method more accessible for research groups that do not have access to conventional loading systems, and could potentially be used in barrier facilities or other remote locations due to the portability of the system. We are making the plans and instructions for the CARD system freely available in the hopes that others will find this system useful for their studies of OA in mice.

Through our use of the CARD system, we found that the system and software are not as user friendly as those of the ELF system. In particular, there was not the same level of real-time feedback on force and displacement. This lack of real-time feedback made it somewhat challenging to set the VCA, and may have contributed to the two failed injuries in the CARD

group. These failed injuries also underline the importance of performing an anterior drawer test or comparable method after loading to confirm injury. Additionally, we found that the loading parameters for the CARD system were not as easily modifiable as those of the ELF system, which may make it necessary for users to make changes in the software in some situations. For example, in studies involving mice with higher body mass, 14 N of compressive force may not be sufficient to induce ACL injury since the injury force has been shown to correlate with body mass [18]. There may also be a desire to modify individual components of the system such as the ankle holster and knee cup, though these changes will be dependent on the specific application.

OA progression following non-invasive knee injury with the CARD system was largely comparable to OA progression following injury with the ELF system. However, there were some indications that injury with the CARD system may have been slightly more severe and led to an accelerated rate of OA progression. In particular, OA progression and synovitis at 2 weeks post-injury were greater for joints injured with the CARD system compared to those injured with the ELF system (though these were not different at week 6). This may reflect the relatively lower amount of control that is inherent in the CARD system, which may have resulted in a greater degree of tissue damage in some knee joint structures in addition to ACL rupture. This may require further investigation and validation in a greater number of samples, though the internal consistency (i.e., variability) in outcomes with the CARD system injuries was not greater than that of the ELF system injuries.

This study has additional limitations that must be acknowledged. First, this study involved a relatively small number of mice (n=5 mice/group/time point), and used only one strain, sex, and

age of mice. Second, this study did not include functional analyses of gait or pain following injury. Third, all injuries were performed using both systems by an experienced used (BAC). It is unclear how easy and effective the CARD system will be for users who do not have previous experience with the ACL-R method. However, despite these limitations, this study described a custom-designed low-cost portable system for ACL-R and provided a validation of the CARD system relative to a commercially available system (ElectroForce 3200) and showed comparable results in post-injury joint laxity, subchondral bone changes, osteophyte formation, synovitis, and OA progression. This novel system could be tremendously useful for some studies of OA in mice, and will provide increased accessibility and utility for the ACL-R method.

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Figure 1:

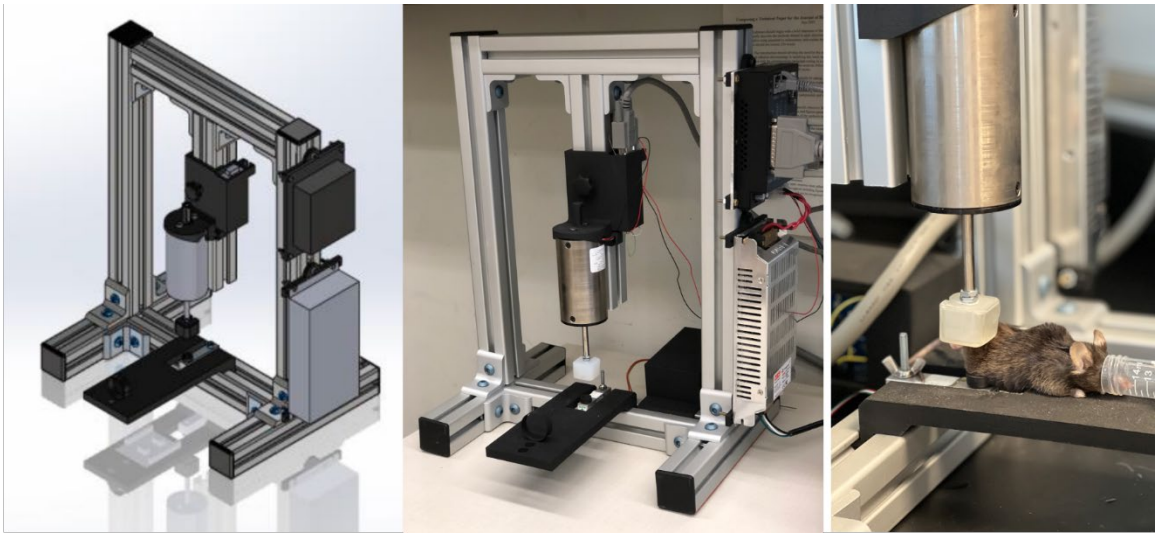


Figure 2:

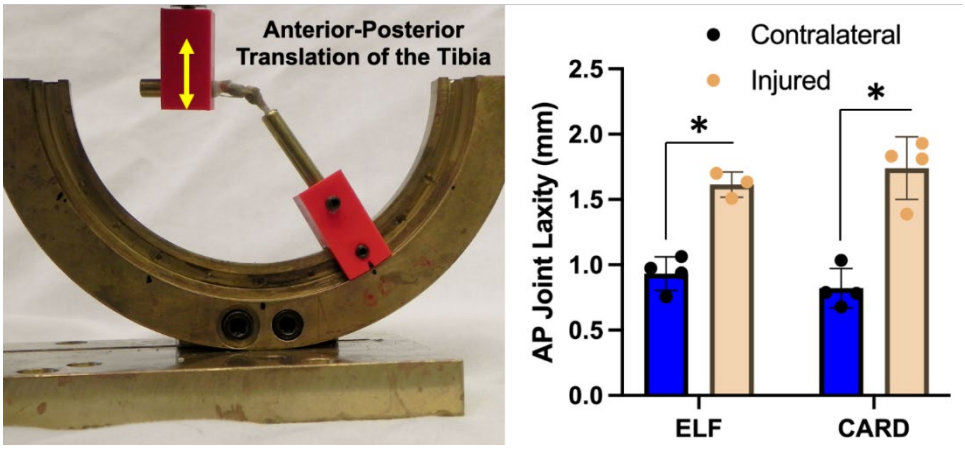

Anterior-Posterior
Translation of the Tibia
Contralateral
Injured
AP Joint Laxity (mm)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
ELF
CARD

Figure 3:

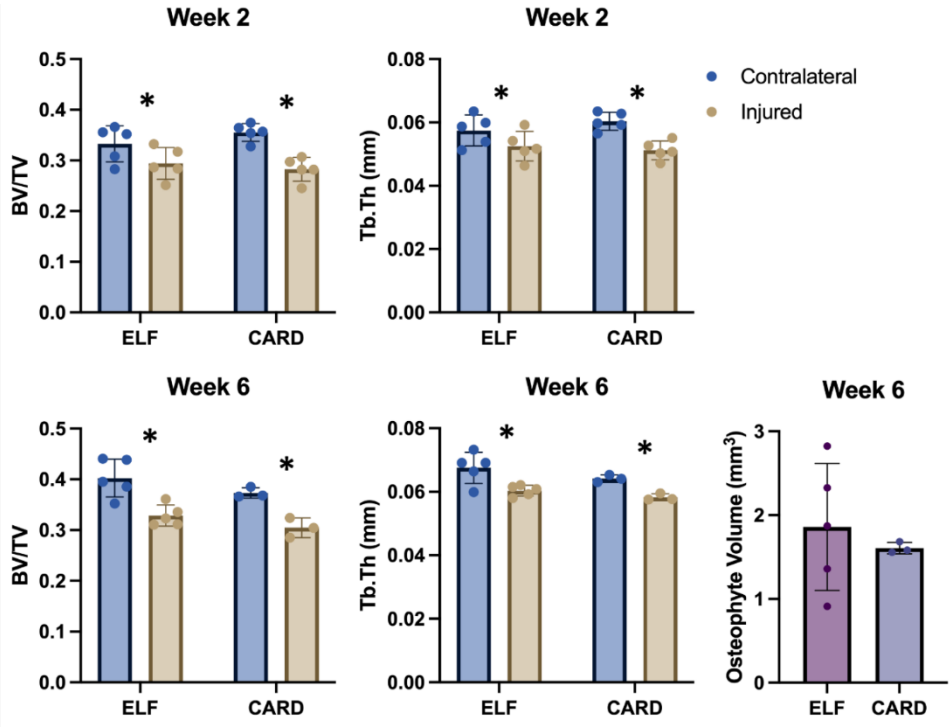

Week 2
Week 2
Contralateral
Injured
BV/TV
Tb.Th (mm)
ELF
CARD
Week 6
Week 6
Week 6
Osteophyte Volume (mm³)

Figure 4:

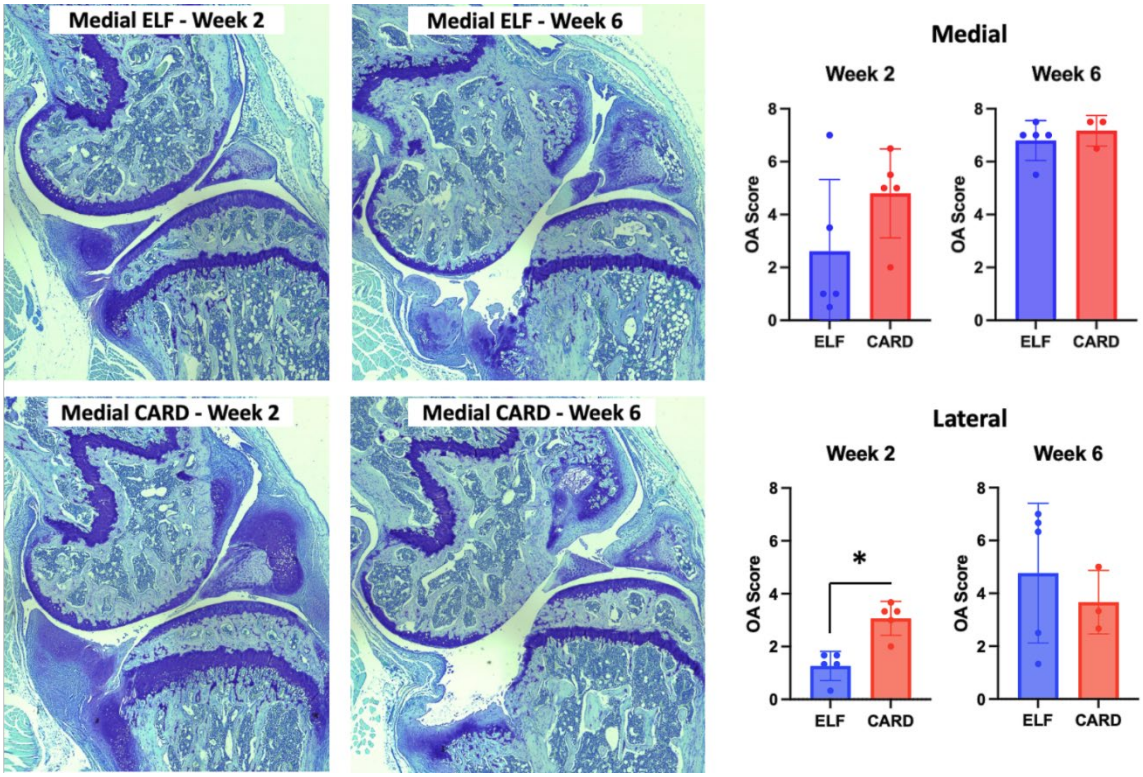
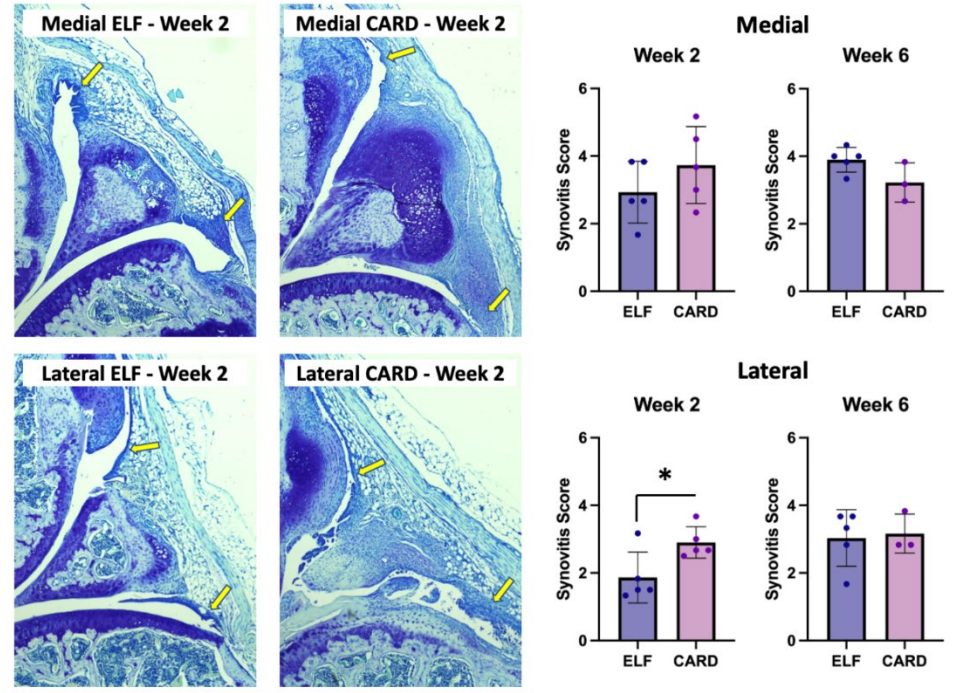

Medial ELF - Week 2
Medial ELF - Week 6
Medial CARD - Week 2
Medial CARD - Week 6
Medial
Week 2
Week 6
OA Score
0
2
4
6
8
ELF
CARD
Lateral
Week 2
Week 6
*

Figure 5:


Medial ELF - Week 2
Medial CARD - Week 2
Lateral ELF - Week 2
Lateral CARD - Week 2
Medial
Week 2
Week 6
Synovitis Score
ELF
CARD
Lateral
*