http://dx.doi.org/10.2106/JBJS.16.00603


# Appendix E-1

## *Methods – Surgical Procedures*

### Chondrocyte Isolation and Propagation

Four weeks prior to scheduled matrix-induced autologous chondrocyte implantation (MACI), cartilage biopsy specimens were obtained arthroscopically from the femoral trochlear ridge of 24 horses that were from 2 to 6 years old. Cartilage biopsy specimens were enzymatically digested and expanded in vitro before seeding on sterile processed collagen type-I/III membranes (Vericel) as described in a pilot study[32]. Passage-2 cells were seeded at 0.5 to 1.0 million chondrocytes per $cm^2$ of collagen membrane and incubated for 48 hours prior to shipping. Additionally, a collagen type-I/III membrane was cultured without seeded chondrocytes for use in control defects implanted with membrane alone.

### Surgical Implantation

The patellofemoral joint contralateral to the joint previously randomly selected for cartilage biopsy was used for the index surgery. Joint fluid was withdrawn and stored for a cell count, protein content, and prostaglandin $E_2$ (PGE2) assay. A 5 to 6-cm miniarthrotomy was made between the middle and lateral patellar tendons as previously described[32]. Briefly, a 15-mm vertical walled defect was made in the proximal aspect of the lateral trochlear ridge, using a skirted custom bit with rigid guide. The guide was initially impacted into the cartilage to create a clean 15-mm-diameter defect with vertical walls. Uncalcified and calcified cartilage was removed down to the subchondral bone. A second defect was formed, 1 cm distal to the initial defect, utilizing limb flexion to expose additional cartilage on the lateral trochlear ridge. Both defects had minimal bleeding after the procedure.

Two 17-mm circular implants were dissected from the 4 × 6-cm chondrocyte-seeded or cell-free collagen type-I/III membrane, taking care to identify the rough surface that had been seeded with chondrocytes. Implantation of the MACI or cell-free collagen membrane was randomly assigned to the proximal and distal defects within the joint for 12 horses, and similarly the proximal or distal position of the MACI membrane was randomized in the second group of 12 horses, in which 1 defect was left ungrafted to heal spontaneously. When a MACI implant or unseeded collagen membrane was utilized, the rough surface was inserted facing downward to juxtapose the subchondral bone. Once in position in the cartilage defect, the edge of the membrane was gently raised and thrombin-activated fibrin (Tisseel; Baxter Healthcare) was injected into the interface between subchondral bone and membrane as illustrated in a previous publication[32]. The edges of the membrane were then gently press fit, and a final seal of fibrin adhesive was injected around the entire perimeter of the membrane. The investigators were blinded as to which membranes were seeded with chondrocytes and which were cell-free. In 3 horses, both defects were left ungrafted.

The arthrotomy layers including synovial membrane, joint capsule, deep fascia, superficial fascia, and skin were sutured, and the horses recovered without limb coaptation.

### Chondrocyte Viability

Residual cell membrane remaining immediately after surgery was assessed for chondrocyte viability using fluorescein diacetate and propidium iodide uptake under high-power confocal microscopy. As a control procedure, cell-laden membranes were snap-frozen at −80°C and assessed using fluorescent confocal microscopy.

### Postoperative Exercise

Exercise was limited to complete stall confinement for the initial 4 weeks after surgery, and then brief walking for an additional 6 weeks before gradual introduction of small paddock exercise.

## *Methods – Histology and Gene Expression*

### Detailed Histologic Methods

The osteochondral samples were fixed in 4% paraformaldehyde, with high-resolution radiographic images obtained to assess subchondral bone structure, and then were decalcified in 10% EDTA (ethylenediaminetetraacetic acid). The samples were then embedded in paraffin and sectioned at 6 μm. Sections were stained with hematoxylin and eosin to evaluate morphology, and toluidine blue was used to assess proteoglycan distribution in the pericellular matrix. Sections for collagen immunohistochemistry were treated with hyaluronidase, and rat anti-bovine type-II collagen primary antibody or rabbit anti-equine type-I collagen primary antibody was applied as described in previous publications[49-51]. Tissue sections were examined in a blinded manner by 2 observers, and a score for defect healing was derived using published parameters to develop a composite score[49-51]. These parameters include scores for defect fill, chondrocyte predominance, chondrocyte cloning, perimeter integration, subchondral bone attachment, extent of tidemark reformation, surface fibrillation, and additional scores for the depth of toluidine blue matrix staining and collagen type-II predominance. The depth of toluidine staining was divided into 4 zones as described[49]. The scores for zones represented staining from <50% (3), 50% to 75% (2), 76% to 90% (1), and >90% to 100% (0). An actual table of the entire scoring system is detailed in a study by Goodrich et al.[49]. These individual scores were summed to develop a final score. Individual scores ranged from 0 (normal) to 3 or 4 (most affected), with a total score ranging from 0 (normal) to 32 (poorest healing). The perfect histologic score, listed as 0, is based on standard normal equine cartilage histologic characteristics. It is not derived from adjacent cartilage or cartilage from the contralateral joint, which was previously used for biopsy to derive chondrocytes for the implant.

### Detailed Gene Expression

Wet tissues from the defects were pulverized in a freezer-mill, and total RNA was isolated and purified. Total RNA was reverse transcribed and amplified using the 1-step quantitative polymerase chain reaction (RT-PCR) technique and the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Expression of aggrecan and collagen type-IIB mRNA were quantitated on repair tissue from all treated joints at 53 weeks. The equine primers and dual-labeled fluorescent probes included the following:

Col IIB: Fwd-CGCTGTCCTTCGGTGTCA
Rev-CTTGATGTCTCCAGGTTCTCCTT
Probe:TCCGGCAGCCAGGACCGAA

ACN: Fwd-GATGCCACTGCCACAAAACA
Rev-GGGTTTCACTGTGAGGATCACA
Probe: CCGAGGGTGAAGCTCGAGGCAA

18S: Fwd-CGGCTTTGGTGACTCTAGATAACC
Rev-CCATGGTAGGCACAGCGACTA
Probe:TCGAACGTCTGCCCTATCAACTTTCGAT

Total copy number of mRNA derived by absolute quantitative PCR was obtained for each gene of interest from a concurrently performed standard curve. These copy numbers were normalized to the nanograms of total RNA, to eliminate the vagaries of RNA-yield per unit weight of cartilage. The copy numbers were then normalized to 18S RNA expression.

## *Methods – Statistical Analysis*

### Statistical Analysis

Comparison of the biochemical and gene expression data from the treated, control, and normal joints was done by analysis of variance (ANOVA), with post-hoc Tukey classification of different groups. Synovial fluid data from implanted joints and contralateral, normal joints were compared at each time point by ANOVA and across time by repeated-measures ANOVA. For ANOVAs with significant F-tests, a Tukey post-hoc procedure was performed to determine which treatment groups were significantly different from each other. Significant interactions of treatment (MACI, cell-free collagen membrane, empty, or contralateral limb control), limb (left and right), or site (proximal or distal) were assessed with follow-up tests. The level of significance was $p < 0.05$. Ordinal data (arthroscopic second-look and gross and histologic scores) were analyzed using Kruskal-Wallis 1-way nonparametric ANOVA when comparing MACI implant, collagen membrane, ungrafted, and paired empty controls. A significant p value on Kruskal-Wallis 1-way nonparametric ANOVA, derived using chi-square approximation, was followed by pairwise follow-up tests using the Kruskal-Wallis all-pairwise comparison. Significant differences between groups were assigned different letters in the data tables and figures.

## *Results – Synovial Fluid PGE2 Formation*

### PGE2 Analysis

Synovial fluid PGE2 levels prior to implantation, at the 12-week second look, and at 53 weeks are presented in Figure 1. PGE2 levels in synovial fluid were not significantly elevated in MACI or cell-free collagen type-I/III membrane-implanted groups at 12 or 53 weeks after surgery compared with the same joints prior to implantation. Levels in paired empty joints increased significantly from the preoperative evaluation to the 12-week sampling. At 53 weeks,

synovial PGE2 levels had decreased to levels similar to those in the preimplantation samples. There were no significant effects of implant type on PGE2 levels at 12 weeks. However, at 53 weeks joints receiving MACI and an empty defect had lower PGE2 levels than joints implanted with MACI and the cell-free collagen membrane.

## Results – Synovial Membrane Histology

### Synovial Membrane Histology and Scoring

Synovial membrane biopsy specimens obtained at the 12-week second-look assessment and 53-week termination were examined and scored for intimal layer thickening, subintimal fibrosis, villus blunting, vascularity, and perivascular lymphoid accumulation. Individual and composite scores for villous architecture are presented in Table VI. At termination, there were few differences between joints receiving MACI and the various controls (Fig. 6). Villous architecture, subintimal fibrosis, intimal thickness, and vascularity were all similar in joints implanted with MACI and cell-free collagen type-I/III membrane, MACI and empty, unimplanted defects, paired empty defects, those previously biopsied contralateral joints, and nonoperatively treated normal joints. Significant differences were evident only for lymphocytic accumulations, with minor perivascular cuffing (Fig. 6). Mean inflammatory cell perivascular infiltrate was 0.75 (with 3 being the maximum) in joints receiving MACI and cell-free collagen membrane, and 0.83 in joints receiving MACI and an empty defect, compared with contralateral, biopsied joints, where mean infiltrate scores were 0.08 and 0.25, respectively, and normal joints, which had a score of zero (Table VI). At 12 weeks, scores for perivascular cuffing were significantly increased in joints receiving MACI or cell-free collagen type-I/III membrane at 12 weeks compared with ungrafted joints.

## *Discussion – Matrix Constituent Response*

### Biochemical Analysis

Biochemical analysis of the repaired cartilage defects indicated that sites treated with a MACI implant had moderate increases in proteoglycan content compared with defects repaired with acellular collagen carrier membrane or those allowed to heal spontaneously. Proteoglycan quantitative assays correlated well with increased toluidine blue histochemically stained matrix evident on histology. Proteoglycan content in MACI-implanted defects had increased to the point where it was not significantly different from normal cartilage evaluated from similar anatomic regions. DNA content of the healing tissue indicated that the defects were more cellular than normal cartilage, but less cellular than healing defects in joints where both defects were allowed to spontaneously heal. Reduced cellularity, more organized histologic characteristics with chondrocyte preponderance and improved collagen type-II content, and improved proteoglycan content are important milestones, especially given the long-term nature of this study and the size of the experimental defects.

## *Discussion*

### The Equine Model

The equine model was selected to provide a critical-sized defect (15 mm) that also provided ample tissue for multiple types of analysis. The equine model is potentially the most closely aligned to the challenges of cartilage healing in man[52]. The thickness of equine cartilage, including the thickness of the calcified cartilage, is similar to that in humans and thicker than that in any other animals typically used for experimental studies of cartilage repair[53]. Critical-sized defects in the equine model are reported to be approximately 6 to 9 mm[54,55], and 12 to 15-mm defects have typically been used in studies of chondrocyte grafting[49,50,56,57]. The biochemical, histologic, and biomechanical characteristics of equine cartilage and repair tissue are also well defined[49,58-60]. These considerations make the equine model sufficiently discriminatory to provide data relevant to human chondrocyte implantation and cartilage repair.

The utilization of two 15-mm defects, separated by only 10 mm, may represent a confounding effect of the control defect on the MACI implant defect. This could be due to inflammation from 1 defect impacting the adjacent defect or a biomechanical insult associated with a pair of defects in close approximation. These factors represent limitations of this study design.

## References

50. Nixon AJ, Begum L, Mohammed HO, I B, O'Callaghan MM, Matthews GL. Autologous chondrocyte implantation drives early chondrogenesis and organized repair in extensive full- and partial-thickness cartilage defects in an equine model. J Orthop Res. 2011 Jul;29(7):1121-30. Epub 2011 Feb 11.
51. Wilke MM, Nydam DV, Nixon AJ. Enhanced early chondrogenesis in articular defects following arthroscopic mesenchymal stem cell implantation in an equine model. J Orthop Res. 2007 Jul;25(7):913-25.
52. McIlwraith CW, Fortier LA, Frisbie DD, Nixon AJ. Equine models of articular cartilage repair. Cartilage. 2011 Oct;2(4):317-26.
53. Frisbie DD, Cross MW, McIlwraith CW. A comparative study of articular cartilage thickness in the stifle of animal species used in human pre-clinical studies compared to articular cartilage thickness in the human knee. Vet Comp Orthop Traumatol. 2006;19(3):142-6.
54. Convery FR, Akeson WH, Keown GH. The repair of large osteochondral defects. An experimental study in horses. Clin Orthop Relat Res. 1972 Jan-Feb;82(82):253-62.
55. Hurtig MB, Fretz PB, Doige CE, Schnurr DL. Effects of lesion size and location on equine articular cartilage repair. Can J Vet Res. 1988 Jan;52(1):137-46.
56. Fortier LA, Mohammed HO, Lust G, Nixon AJ. Insulin-like growth factor-I enhances cell-based repair of articular cartilage. J Bone Joint Surg Br. 2002 Mar;84(2):276-88.
57. Sams AE, Nixon AJ. Chondrocyte-laden collagen scaffolds for resurfacing extensive articular cartilage defects. Osteoarthritis Cartilage. 1995 Mar;3(1):47-59.
58. Fortier LA, Balkman CE, Sandell LJ, Ratcliffe A, Nixon AJ. Insulin-like growth factor-I gene expression patterns during spontaneous repair of acute articular cartilage injury. J Orthop Res. 2001 Jul;19(4):720-8.
59. Strauss EJ, Goodrich LR, Chen CT, Hidaka C, Nixon AJ. Biochemical and biomechanical properties of lesion and adjacent articular cartilage after chondral defect repair in an equine model. Am J Sports Med. 2005 Nov;33(11):1647-53. Epub 2005 Aug 10.
60. Gratz KR, Wong VW, Chen AC, Fortier LA, Nixon AJ, Sah RL. Biomechanical assessment of tissue retrieved after in vivo cartilage defect repair: tensile modulus of repair tissue and integration with host cartilage. J Biomech. 2006;39(1):138-46. Epub 2004 Dec 30.