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Materials and Methods

Horses: Physical and Radiographic Examinations

Twelve horses were acquired and physical examinations were performed to assess for lameness or health issues prior to entrance into the study. Both stifles of all horses were radiographed and confirmed to have no abnormalities. The horses were isolated for two weeks prior to the study and trained to walk in hand in anticipation of a postoperative exercise regimen.

Preparation of Bone Marrow Aspirate: Isolation and Expansion of Bone-Marrow-Derived Mesenchymal Stem Cells (BMDMSCs)

Bone marrow was aspirated from the ilium, aliquoted into centrifuge tubes, and spun at 100 × g for five minutes. The bone marrow plasma was collected down to the interface of the red blood-cell pellet and then centrifuged at 1000 × g for ten minutes to pellet the nucleated cells. The nucleated cells were resuspended into low-glucose Dulbecco modified Eagle medium (Invitrogen) containing 10% fetal bovine serum (Hyclone), 25 mM HEPES (Invitrogen), and antibiotic-antimycotic solution (Invitrogen) and seeded into tissue culture flasks at a density of 0.267×10^6 cells/cm^2. The medium was changed after twenty-four hours to remove nonadherent cells. After an additional six to seven days of culture, colony-forming MSCs were harvested using 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) (Invitrogen), counted, and reseeded into monolayer cultures at 10×10^3 cells/cm^2 in proliferation medium consisting of alpha minimal essential medium (Invitrogen) plus 10% fetal bovine serum and 2 ng/mL of fibroblast growth factor-2 (R&D Systems) for two days. The MSCs were collected with 0.25% trypsin/EDTA, reseeded at 0.8×10^3 cells/cm^2 in proliferation medium, and expanded for four to five days. Multilineage potential was not confirmed, but when we previously used cells from this isolation technique we identified chondrogenic and osteogenic potential, and cell surface markers, that are consistent with MSCs (typically twenty of twenty donor samples are confirmed as chondrogenic and osteogenic using these methods)[1,2]. For each horse, MSCs were cryopreserved in autologous 95% serum plus 5% dimethyl sulfoxide (DMSO; Sigma-Aldrich) in a freezing container at −80°C and then stored in liquid nitrogen. The day before surgery, cells were thawed and plated at 10,000 cells/cm^2 in culture medium. On the day of implantation, cells were trypsinized and washed three times in phosphate buffered saline solution (PBS) and combined with the fibrinogen portion of the fibrin glue.

Preparation of Autologous Platelet-Enhanced Fibrin (APEF)

Blood was collected from each animal into a 450-mL-capacity acid citrate dextrose (ACD) blood bag containing 63 mL of CPDA-1 (Jorgensen Laboratories), whereupon it was sterilely transferred into conical tubes. The tubes were centrifuged at 200 × g for eighteen minutes to pellet the red and white blood cells. Plasma was transferred into new tubes and stored at −80°C until the day of surgery. Frozen plasma samples were thawed overnight at 4°C. On the day of surgery, blood was again collected into an ACD blood bag as described above and centrifuged as described above. Plasma was transferred into a new tube, and platelets were pelleted with centrifugation at 1000 × g for ten minutes. Platelet pellets were suspended in 1 mL of thawed plasma. Then 800 μL of cold 100% ethanol was added to a 5-mL aliquot of thawed plasma and incubated on ice for thirty minutes. Samples were centrifuged at 1000 × g for fifteen minutes. Fibrinogen pellets were suspended in an equal volume of platelet-poor plasma, and 4×10^9 platelets were added per 2 mL of fibrinogen. Forty million BMDMSCs were added to the 2 mL of fibrinogen for the APEF+BMDMSCs group. All defects received APEF consisting of 1 billion platelets per mL (approximately five times the baseline platelet count in blood), with the addition of 10 million BMDMSCs per mL for defects assigned to the APEF+BMDMSCs group. All defects were filled with approximately 1 mL of the biologic milieu.

Arthroscopic Defect Creation and Defect Grafting

A customized reamer was used to create a 15-mm round chondral defect arthroscopically on the lateral trochlear ridge of both femoropatellar joints. In each horse, one defect was randomly assigned to be treated with APEF+BMDMSCs and the contralateral defect was treated with APEF alone. Briefly, horses were positioned in dorsal recumbency and an arthroscopic portal was placed between the middle and lateral patellar ligaments halfway between the patella and the tibial crest. A separate instrument portal was made medially, medial to the middle patellar ligament to facilitate lifting of the joint capsule with a straight metal instrument from the medial aspect to the lateral aspect of the joint. The femoropatellar joint was arthroscopically examined, after which a 4-cm incision was made just over the trochlear ridge, through the skin, fascia, femoropatellar fat pad, and joint capsule. A defect was made using a 15-mm-diameter drill-bit placed through a cannula that had a stop mechanism that allowed the drill to remove only 2 mm of cartilage depth down to, but not including, the subchondral bone. This cannula and obturator were inserted through the incision and placed on the lateral trochlear ridge of the femoropatellar joint. The 15-mm-diameter drill-bit was then inserted through the cannula, and the cartilage was removed with the drill while continuous arthroscopic lavage of the joint was performed. When the obturator was removed, the round chondral defect and exposed subchondral bone were observed and any existing calcified cartilage was removed using a curet. The joint was then dried with CO_2 gas arthroscopically delivered through the cannula. A needle was attached to a Duploject syringe (Baxter) and was then positioned over the defect as viewed arthroscopically. The APEF or APEF+BMDMSCs was then delivered using the Duploject syringe, with the mixture in one of the two syringes that make up the device and bovine thrombin (Sigma-Aldrich) in the other. The scaffold was allowed to set up under arthroscopic observation and CO_2 gas. Following several minutes of drying, the fibrin scaffolds contracted to the point that the peripheral cartilage surrounding the defect was even with the surface of the scaffold. Arthroscopic images were recorded. The joint was then redistended with fluid, and the arthroscopic portals were closed using a simple interrupted suture and a stent placed over the lateral-most incision.

Postsurgical Exercise Regimen

Following creation of the surgical defect, the horses were fully weight-bearing and mobile. The exercise protocol consisted of stall rest for six weeks, twice-daily walks for five minutes for weeks seven to nine, twice-daily walks for ten minutes for weeks ten to twelve, stall rest (following second-look arthroscopy) for weeks thirteen to fifteen, and free exercise in a small (approximately 0.33-acre) paddock for weeks sixteen to fifty-six.

Arthroscopic Examination at Three Months After Defect Creation

Arthroscopic examination was performed three months following defect creation and scaffold placement. Briefly, an arthroscopic portal was created between the lateral and middle patellar ligaments and the instrument portal was created between the middle and medial patellar ligaments. The defects were gently palpated with a right-angled probe to determine the hardness of the tissue and the attachment of the tissue to the surrounding peripheral cartilage. All defects were photographed and graded by three surgeons (L.R.G., C.W.M., and C.R.C.) who were blinded to the treatment group[3] (Table I).

Post-Mortem Examinations

Arthroscopy

The horses were euthanized twelve months following defect creation. The stifles were excised and shipped on ice to a facility for MRI analysis and arthroscopic examination. Arthroscopic examinations were performed as described above (Table I). All defects were again gently probed with an arthroscopic instrument, photographed, and graded by two surgeons (L.R.G. and C.R.C.) who were blinded to the treatment group.

MRI

Two MRI sequences were acquired on intact stifle joints for morphological evaluation. 2D axial proton density (PD) turbo spin echo (TSE) images with fat saturation (FS) were acquired with a TR/TE of 5000/47 ms, echo train length (ETL) of 11, and 2 averages, with a 12-cm field of view (FOV) and 384 matrix for 313 × 313-μm in-plane resolution and 3-mm slice thickness. A 3D axial PD SPC RST image was acquired with a TR/TE of 1200/45 ms, ETL of 67, 2 averages, 120° flip angle, 16-cm FOV, and 320 matrix for 0.5 × 0.5-mm in-plane resolution and 0.6-mm slice thickness. All MRIs were evaluated in multiple categories by a radiologist blinded to the treatment group[4] (Tables II and III).

Quantitative MRI (qMRI) T2 Mapping of Equine Explants

Osteochondral explants consisting of the lateral trochlear ridge of equine femoropatellar joints were imaged with qMRI standard T2 mapping using a 3-T human clinical MRI scanner (MAGNETOM Trio Tim, Siemens Medical Solutions) with an eight-channel knee coil (Invivo). Nylon screws attached the specimen, mounted on an adaptor plate, to a registration plate with MRI lucent markers used for image orientation and location referencing. Specimens were seated on a saline-solution bag for proper MRI coil loading and then centered in the coil, oriented such that the proximal-distal axis of the explant was aligned superiorly-inferiorly (parallel to the bore of the magnet), with the anterior surface of the lateral trochlear ridge facing up (anterior-posterior). Localizer sequences and iterative positioning adjustment of the specimens were performed until orthogonal localizer images demonstrated that the pattern of MRI lucent wells in the registration plate was in line with the axial, coronal, and sagittal planes of the imaging slab.

Axial T2-weighted images were acquired with a multi-slice multi-echo spin-echo sequence with seven echoes (TE of 11, 22, 33, 44, 55, 66, and 77 ms) and a TR of 1800 ms. Images were acquired with 313 × 313-μm^2 in-plane resolution, 2-mm slice thickness, and a 2-mm gap between slices. Scan time was eleven minutes and thirty-two seconds for all seven images. The sequence was acquired twice. For the second acquisition, the imaging slab was shifted 2 mm in order to image the tissue in the gaps between the slices of the first acquisition. Following MRI, explants were frozen at −20°C and shipped to another location for indentation testing and micro-CT.

qMRI Image Processing

T2 maps were calculated with a mono-exponential pixel-by-pixel T2-fit routine using MRI Mapper software (Beth Israel Deaconess Medical Center and MIT [Massachusetts Institute of Technology]) running on a MATLAB platform (The MathWorks). A single axial slice from the center of each cartilage repair was evaluated. A region of interest was manually drawn on each fitted map to encompass the full-thickness depth and entire width of the cartilage repair (approximately 1 to 10 pixels deep by approximately 50 pixels wide—i.e., full depth × 1.5 cm). All qMRI T2 maps were evaluated and scored[5]. T2 values were recorded for each region of interest.

Sample Registration for Indentation Testing, Micro-CT Imaging, Tissue Thickness, and Histological Analysis

A transparent overlay was placed over the trochlear lesion to indicate biomechanical test sites, placement of micro-CT markers, and histological borders (Figs. 1-A and 1-B). Lesion dimensions were measured in the proximal-distal and medial-lateral directions, and an overlay based on the geometry was created. The overlay was centered on the repair site, with the remaining portions of the overlay allowed to fit the contour of the trochlear surface, and anchored with dissecting pins. Then holes (0.060 in [1.524 mm] in diameter and 2 to 3 mm deep) that served as micro-CT registration markers were drilled at defined locations relative to the center of the repair, perpendicular to the registration plate. During the drilling process, samples were kept moist with PBS supplemented with proteinase inhibitors. The markers allow micro-CT image data to be registered relative to the registration plate and also serve as the reference guide for histological processing.

Indentation Testing

Cartilage load-bearing function was mapped at fifteen sites (five regions) (Fig. 1-C) on each trochlea (one to four measurements per site), for a total of thirty-six measurements per trochlea. These sites were in the lesion center (central repair region; sites 1 through 5), the lesion edge (peripheral repair region; sites 6 through 9), adjacent to the lesion (adjacent host region; sites 10 through 13), near the lesion (near host region; site 14), and far from the lesion (far host region; site 15), with the last two regions serving as controls. Briefly, the sample was thawed and kept hydrated in PBS plus protease inhibitors. The sample was then held in a clamp that allowed for alignment of the indenter tip to the cartilage surface as well as positional indexing. At each site, rapid indentation tests were performed for one second to a depth of 100 μm using a 0.8-mm-diameter sphere-ended indenter attached to a Mach-1 V500cs mechanical testing system (Biomomentum) to allow measurement of load and determination of structural stiffness (force per indentation depth)[6]. In one animal, the far distal control site was not available for indentation measurements, so a far proximal control site was used. Following indentation testing, samples were frozen until imaging with micro-CT.

Micro-CT Imaging

Micro-CT imaging was performed to visualize the cartilaginous repair tissue and bone structure relative to the indentation test sites and used to quantify cartilaginous repair tissue thickness[6]. Samples were thawed in PBS plus protease inhibitors and imaged on a model-1076 micro-CT scanner (SkyScan, Belgium), inside a low-density polyethylene container, prepared with a PBS-moistened Kimwipe (Kimtech) to provide a humidified environment for the sample during scanning. Imaging was performed at $(36\ \mu m)^3$ isotropic voxel size[8], by applying an electrical potential of 100 kVp and a current of 100 μA, and using a 0.038-mm copper plus 0.5-mm aluminum filter. A beam-hardening correction algorithm was applied during image reconstruction of every scan. Image slices were selected and analyzed using SkyScan's Dataviewer. In addition, a custom program was used to create a series of 2D transaxial, sagittal, and coronal slices. Images were rotated such that the proximal and distal holes were on the central sagittal plane. Similarly, transaxial images were obtained by first selecting the cartilage's surface at the center point

between the drill-holes. After the images had been positioned in the sagittal and transaxial orientation, the coronal image was selected at the center point between the drill-holes.

Matrix fixed charge density in the cartilaginous repair tissue was assessed with micro-CT enhanced with Hexabrix (ioxaglate meglumine and ioxaglate sodium; Guerbet), as previously described[7,8]. Hexabrix distributes inversely to the fixed charge density (proteoglycan content) in soft tissues, and is a sensitive (inverse) indicator of proteoglycan content[9]. After structural imaging, samples were fixed in 4% paraformaldehyde for five to seven days, rinsed with PBS, and finally placed in 20% Hexabrix in PBS plus protease inhibitor solution for forty-eight to seventy-two hours, after which they were scanned at $(18\ \mu m)^3$ voxel size, at the same electrical setting. The Hexabrix-micro-CT gray value (i.e., x-ray attenuation) within the cartilaginous repair tissue was calculated. For calibration, tubes containing 0%, 5%, 10%, 15%, and 20% Hexabrix in the equilibration solution were imaged alongside the samples.

Tissue Thickness

Cartilaginous repair tissue thickness corresponding to each of the fifteen test sites was determined from micro-CT scans as the height from the tissue surface to the bone-tissue interface, essentially as described previously[6]. Briefly, from sagittal and transaxial slices, each image was thresholded to determine tissue-air and tissue-bone interfaces. The surface contours were determined so that vectors perpendicular to the horizontal orientation could be used to determine tissue thickness at discrete locations. At each site, a test width of 0.8 mm was used to determine the area-averaged thickness.

Histological Analysis

Cartilaginous repair tissue was analyzed histologically. Samples were harvested from the lesion and far control sites, decalcified in 10% EDTA, embedded in paraffin, sectioned at 8 μm, deparaffinized, stained with hematoxylin and eosin or safranin O, and digitized. All histological sections were graded according to a modified O'Driscoll scoring system (Table IV) as previously described[10].

Data Reduction and Analysis

Gross images were evaluated to determine if an indentation measurement was made on a site devoid of cartilaginous repair tissue (Figs. 1-A and 1-B). A measurement within a test site that fell into this category was excluded from indentation stiffness analysis. Of 192 total measurements, eight measurements from five trochleae originating from four horses were excluded.

From the biomechanical data, the peak load was normalized to the applied 100-μm indentation displacement to determine structural stiffness (in N/mm). When cartilage thickness was calculated, the peak load was normalized to the cross-sectional area of the indenter and displacement was normalized to the cartilage thickness of the 0.8-mm test width to determine material stiffness (in MPa).

Data from sites 1 through 5 were averaged to provide the thickness values and Hexabrix-enhanced micro-CT results for the central region of the lesion (Fig. 1-C). Data from sites 6 through 9 were averaged with neighboring sites 10 through 13 to provide the thickness values and Hexabrix-enhanced micro-CT results for the defect edge (Fig. 1-C). For the measurements of the structural stiffness and material stiffness, individual measurements at each site were divided into those in the interior of the lesion (edge) and those exterior (adjacent) to the lesion (Fig. 1-C). Data at sites 14 and 15 were each individually averaged for control articular cartilage tissue values. Data are given as the mean and standard deviation of the mean.

Statistical Analysis

Scores derived from the arthroscopic, histological, MRI, and biomechanical evaluations were compared using the Wilcoxon signed rank analyses with significance set at $p < 0.05$. All analyses were performed with and without the horses in which bone formed within the defects. ■

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