PROTOCOLS ARTICLE

A Rabbit Femoral Condyle Defect Model for Assessment of Osteochondral Tissue Regeneration

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Osteochondral tissue repair represents a common clinical need, with multiple approaches in tissue engineering and regenerative medicine being investigated for the repair of defects of articular cartilage and subchondral bone. A full thickness rabbit femoral condyle defect is a clinically relevant model of an articulating and load bearing joint surface for the investigation of osteochondral tissue repair by various cell-, biomolecule-, and biomaterial-based implants. In this protocol, we describe the methodology and 1.5- to 2-h surgical procedure for the generation of a reproducible, full thickness defect for construct implantation in the rabbit medial femoral condyle. Furthermore, we describe a step-by-step procedure for osteochondral tissue collection and the assessment of tissue formation using standardized histological, radiological, mechanical, and biochemical analytical techniques. This protocol illustrates the critical steps for reproducibility and minimally invasive surgery as well as applications to evaluate the efficacy of cartilage and bone tissue engineering implants, with emphasis on the usage of histological and radiological measures of tissue growth.

Keywords: full thickness defect, osteochondral, femoral condyle, rabbit model

Impact Statement

Although multiple surgical techniques have been developed for the treatment of osteochondral defects, repairing the tissues to their original state remains an unmet need. Such limitations have thus prompted the development of various constructs for osteochondral tissue regeneration. An *in vivo* model that is both clinically relevant and economically practical is necessary to evaluate the efficacy of different tissue engineered constructs. In this article, we present a full thickness rabbit femoral condyle defect model and describe the analytical techniques to assess the regeneration of osteochondral tissue.

Introduction

OSTEochondral defects refer to conditions where a segment of cartilage, along with a portion of the underlying subchondral bone, resorbs and eventually produces a lesion within the joint surface.¹ The cartilage layer does not naturally heal because of its unique avascularity and low cell density and, if left untreated, eventually progresses into conditions such as osteoarthritis.² Various surgical methods for cartilage repair have thus been developed such as microfracture, osteochondral autograft/allograft replacement, and autologous chondrocyte implantation.^{3,4} Nevertheless,

these surgical interventions have been shown to result in the regeneration of fibrous cartilage, which lacks the mechanical properties seen in healthy articular cartilage.^{5,6}

Tissue engineering provides a promising alternative to overcome these challenges by combining patient-specific cells, bioactive molecules to guide the cells' behavior and differentiation lineage, and scaffolds to deliver these components into the defect site. To validate the clinical feasibility of tissue engineered constructs developed and analyzed *in vitro*, it is mandatory in the regulatory context to conduct further *in vivo* studies, using animal models to provide proof of principle. In choosing an animal model, it

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is important to consider both the clinical relevance of the defect model and the logistical feasibility of conducting a sufficiently large-scale study to draw statistically relevant conclusions. In the case of an osteochondral defect model, the ability to generate a full thickness defect on a load-bearing surface of the articular cartilage is also required.

The aim of our project was to describe the use of a rabbit medial femoral condyle defect model as a platform to generate and study the regeneration of full thickness osteochondral defects, which are defined as an osteochondral defect that extends to the cancellous bone underneath the cartilage and the subchondral bone layer.^{7,8} This animal model is validated by guidelines in ASTM F2451-05: Standard Guide for *In Vivo* Assessment of Implantable Devices Intended to Repair or Regenerate Articular Cartilage, which also describes the usage of other animal models such as ovine and porcine models.⁹ Compared with smaller rodent models such as rats, the rabbit model offers a relatively larger joint surface and thicker cartilage layer for implantation of materials.^{9,10} In addition, smaller rodent models offer less clinical relevance compared with rabbits, as murine growth plates do not close with skeletal maturity.^{10,11} Large animals such as ovine and porcine models offer a thicker cartilage layer compared with the rabbit model, but suffer from high maintenance costs, reducing feasibility for large scale *in vivo* studies.^{9,10} In modeling subchondral bone, studies have additionally established greater compositional similarity in bone mineral density and bone volume fraction between rabbit and human tissue, relative to other common large animal models such as horses and sheep.^{12,13} Rabbit models thus provide benefits over both small rodent and large animal models by providing a clinically relevant articulating joint surface that is sufficiently large for full thickness defects, while also being economically feasible.

In this study, we describe the surgical protocol for the generation of full thickness defects on the load-bearing and articulating surface of rabbit femoral condyles, and several methods to analyze the degree of tissue regeneration. This model has been widely utilized to analyze the *in vivo* regenerative capacity of tissue engineered constructs developed and validated *in vitro*. Specifically, the use of this *in vivo* model has been validated for the delivery of:

1. Cell-seeded/encapsulated scaffolds and hydrogels fabricated from synthetic, biocompatible polymers and biological materials.
2. Multilayered constructs designed to mimic the cartilage and the subchondral bone layers.
3. Patient-specific porous scaffolds fabricated using three-dimensional (3D) printing techniques.
4. Cells suspended in a biological carrier/gel or as a pellet.

Overview of the Procedure

For the generation of the defect, an incision is first made through the skin, fascia, and joint capsule on the medial side of the knee joint. The patellar tendon is then luxated laterally and the knee is flexed to expose the articular surface of the medial femoral condyle. Using a dental drill, a full thickness cylindrical defect is then generated in a stepwise

manner until the desired defect diameter is reached. After construct implantation, the knee is extended and the patella is reduced to its original location before closing the wound site in anatomical layers. After the duration of the experiment, the animal is killed for tissue harvest. The isolated femoral condyle can then be analyzed by various techniques including histology, microcomputed tomography (MicroCT), biochemical assays, and mechanical testing.

Experimental Design

Implantation material

Constructs fabricated from various materials can be implanted in the defect site, provided their toxicity and cytocompatibility have been validated *in vitro*. Hydrogels are widely used for their ability to encapsulate and deliver a large number of cells. They can be fabricated from both synthetic^{14–17} and biological^{18–21} materials, and can also be used to deliver biomolecules to provide the necessary signals for the encapsulated cells.^{15,17} Hydrogels can also be acellular, in which case the defect model can be used to study the regenerative property of the construct material.²⁰ In addition to hydrogels, porous scaffolds provide another means to deliver cells and biomolecules to the defect site.^{22–24} The scaffolds are most often preseeded with cells to give cells sufficient time to adhere to the surface. With the advent of additive manufacturing technology, scaffolds that have been 3D printed to match the size and shape of the defect site have also been implanted.^{25–27} In addition, multilayered constructs have been designed to better mimic the osteochondral architecture. Such constructs can be either composed of two materials with different mechanical and biological properties,^{25,26} or fabricated from the same material but with different properties (different porosities, loaded biomolecules, etc.) to distinguish the cell behavior in the two layers.^{15,23} Last but not least, scaffold-free cell pellets can also be delivered into the defect site.²⁸ To provide an example of an implantable construct, this protocol describes the preparation of an acellular or cell-encapsulated oligo(poly(ethylene glycol) fumarate) (OPF)-based hydrogel system previously investigated in our laboratory.¹⁴

Cell sources

Mesenchymal stem cells (MSCs) are the most widely used cell type for fabricating cell-laden osteochondral constructs. In addition to being able to undergo osteogenic and chondrogenic differentiation,²⁹ MSCs are relatively non-immunogenic and have been used for allogeneic implantations.^{30,31} Xenogeneic implantations using MSCs isolated from humans have also been reported.^{32,33} Moreover, articular chondrocytes, both autologous³⁴ and allogeneic,^{28,35} have been used as cell-sources for this model without eliciting immunogenic effects.

Implantation time

Various time courses of the *in vivo* study have been implemented, and there is no consensus on what the optimal study duration should be.^{10,14,26} Rather, the limitations are placed by logistical concerns such as the number of animals, housing space, and study groups. In general, time points between 4 and 12 weeks are most common and can

represent short- to mid-term tissue regeneration, although studies with shorter (2 weeks) and longer (48 weeks) end points have also been reported.^{10,14,15,26}

Defect

Cylindrical defects with varying diameters and depth can be generated on the surface of the femoral condyle. Defects can also be generated bilaterally to reduce the number of rabbits utilized in a study.^{10,14} The geometries of the defect can range from 2 to 5 mm in diameter, and 1–5 mm in depth. Irregular-shaped full thickness defects have also been implemented, but they are much less common.¹⁸ The size of the defect depends mostly on the skeletal maturity of the rabbit, where larger defects can be generated in older rabbits with wider femoral condyle surface. In addition, generating a defect that is too deep can cause the marrow to leak and thus should be avoided unless it is a necessary step in the procedure (e.g., microfracture of the subchondral bone³⁶).

Materials Required

Animal species and selection

Skeletally mature (i.e., 6 months old) New Zealand White (NZW) rabbits were used for this protocol. The NZW strain of rabbit is predominantly utilized in osteochondral research because of its favorable behavioral characteristics and fewer health problems, although other strains such as Dutch Belted rabbits have been infrequently utilized as well.^{37,38} Skeletally immature rabbits (<6 months old) as young as 9 weeks old have also been used in models of osteochondral repair, but are known to exhibit much more rapid rates of skeletal change and turnover of both bone and cartilage.^{10,39} Given sex-related differences in bone density, cartilage thickness, and the regulation of chondrogenic and osteogenic processes,^{10,40,41} it is recommended to utilize both male and female rabbits in study design. In the rabbit model, sex-related hormones have been directly implicated in the regulation of chondrocyte proliferation and maturation, underscoring the necessity to include both sexes when studying osteochondral regeneration.⁴¹ In terms of sample size, earlier studies in our laboratory have utilized 12 implants, that is, 12 defect sites, for *in vivo* studies with randomized bilateral implantation, but power analyses should be conducted in each case to calculate an appropriate sample size.^{14,15,42,43} All animal procedures described here are in accordance with protocols approved by the Institutional Animal Care and Use Committee and in agreement with the animal care and use guidelines set forth by the National Institutes of Health.

Personnel and expertise

This protocol can be performed by a team consisting of, at minimum, two researchers serving as primary surgeons and one veterinary technician trained in the administration and monitoring of anesthesia. The two researchers serving as primary surgeons should be trained in basic rabbit anatomy, handling, subcutaneous drug administration, and basic surgical techniques. It is strongly recommended to have an additional assistant or technician present to assist with nonsterile equipment and supplies. Nonsterile steps in this protocol can be performed by either the veterinary techni-

cian, if not otherwise occupied, or the recommended non-sterile assistant. All personnel should have appropriate institutional training and approval.

Equipment and tools

1. Autoclave (Tuttnauer 2540 M).
2. Vital signs monitor for ECG, NIBP, rectal temperature, and pulse oximeter (DRE ASM 5000).
3. Pulse oximeter for capnography (Nonin 9847 V).
4. Rectal temperature probe (cat. no. 07-870-2519; Patterson Veterinary).
5. Small animal anesthesia machine (Matrx VMS).
6. Veterinary anesthesia ventilator (Hallowell EMC Model 2002).
7. Warming pad (HTP-1500).
8. Orbital shaker table (IKA KS 501).
9. Electronic calipers (cat. no. 8647A51; McMaster-Carr).
10. Electric clippers (Oster Volt).
11. Dental drill and contra-angle (NSK Surgic XT Plus).
12. Adson toothed forceps (cat. no. 07-803-4538; Patterson Veterinary).
13. Curved mosquito forceps (cat. no. 07-803-4090; Patterson Veterinary).
14. Double-ended retractor (cat. no. 07-809-8466; Patterson Veterinary).
15. Stainless steel spatula (cat. no. 21-401-10; Fisher Scientific).
16. Suture scissors (cat. no. 07-803-4470; Patterson Veterinary).
17. Needle drivers (cat. no. 07-803-4231; Patterson Veterinary).
18. A 2.0×8.5 mm stainless steel drill bit (cat. no. 200-85; Osseo Scientific).
19. A 2.75×8.5 mm stainless steel drill bit (cat. no. 275-85; Osseo Scientific).
20. A 3.0×8.5 mm stainless steel drill bit (cat. no. 300-85; Osseo Scientific).
21. A 1/4" outer diameter, 1/8" inner diameter polytetrafluoroethylene (PTFE) tubing (cat. no. 8547K23; McMaster-Carr).
22. A 1/4" outer diameter, 1/16" inner diameter PTFE tubing (cat. no. 8547K22; McMaster-Carr).
23. Sandpaper, 40 grit (cat. no. 4673A76; McMaster-Carr).
24. Bone rongeur (cat. no. 07-803-3900; Patterson Veterinary).
25. Diamond Saw (cat. no. 650; South Bay Technology).
26. MicroCT Scanner (SkyScan 1172).
27. Microtome (Leica RM2165).

Reagents and surgical materials

1. E-Z scrub brush (cat. no. 07-804-3107; Patterson Veterinary).
2. Sterile gown, gloves, facemask, head cover, and shoe covers (Patterson Veterinary).
3. Endotracheal tube, 3.0 cuffed (cat. no. 07-872-2111; Patterson Veterinary).
4. Suction canister (cat. no. 07-892-7708; Patterson Veterinary).

5. Suction line and handle (cat. no. 07-811-8112; Patterson Veterinary).
6. Sterile disposable scalpels No. 15 (cat. no. 07-800-3549; Patterson Veterinary).
7. Alcohol pads (cat. no. 07-839-8871; Patterson Veterinary).
8. Chlorhexidine solution (2%) (cat. no. 07-892-4243; Patterson Veterinary).
9. Absorbent underpads (cat. no. 56617-006; VWR).
10. Surgical drapes (cat. no. 07-802-5000; Patterson Veterinary).
11. Surgical towels (cat. no. 07-810-8618; Patterson Veterinary).
12. Nonwoven gauze sponges (cat. no. 07-847-3539; Patterson Veterinary).
13. A 22G×1" intravenous catheter (cat. no. 07-893-0804; Patterson Veterinary).
14. Winged infusion set (cat. no. 07-869-1251; Patterson Veterinary).
15. Povidone iodine swabsticks (cat. no. 07-858-2019; Patterson Veterinary).
16. Sterile 1 mL syringes (cat. no. 14-823-30; Fisher Scientific).
17. Sterile 3 mL syringes (cat. no. 14-823-435; Fisher Scientific).
18. Sterile 60 mL syringes (cat. no. 14-955-461; Fisher Scientific).
19. 16G×1" needles (cat. no. 14-826-18A; Fisher Scientific).
20. 23G×1" needles (cat. no. 14-826-6B; Fisher Scientific).
21. 3-0 Vicryl sutures (cat. no. 07-807-0534; Patterson Veterinary).
22. 3-0 Monocryl sutures (cat. no. 07-809-9540; Patterson Veterinary).
23. 4-0 Vicryl sutures (cat. no. 07-808-9814; Patterson Veterinary).
24. Tissue adhesive (3 M; cat. no. 70200742529).
25. Pull-on pajama pants, 3–6 months old (cat. no. B07FK77JL6; Amazon).
26. Vetrap bandaging tape (cat. no. 07-805-5023; Patterson Veterinary).
27. Surgical ruler (cat. no. 07-849-0214; Patterson Veterinary).
28. Isopropyl alcohol (cat. no. 07-801-3514; Patterson Veterinary).
29. Isoflurane (cat. no. 07-893-1389; Patterson Veterinary).
30. Oxygen USP grade (AirGas).
31. Ketamine (cat. no. 07-803-6637; Patterson Veterinary).
32. Acepromazine (cat. no. 07-893-5734; Patterson Veterinary).
33. Buprenorphine (cat. no. 07-892-5235; Patterson Veterinary).
34. Bupivacaine (cat. no. 07-890-4881; Patterson Veterinary).
35. Lactated Ringer's solution (LRS) or physiological saline solution (cat. no. 07-892-7201; Patterson Veterinary).
36. Rimadyl® (carprofen) (cat. no. 07-844-7425; Patterson Veterinary).
37. Baytril® (enrofloxacin) (cat. no. 07-891-6228; Patterson Veterinary).
38. Euthanasia solution (cat. no. 07-867-9022; Patterson Veterinary).
39. Tissue specimen containers (cat. no. 16-320-730; Fisher Scientific).
40. 10% neutral-buffered formalin (cat. no. HT501128; Sigma-Aldrich).
41. 200 proof ethanol (cat. no. 89125-188; VWR).
42. EDTA (ethylenediaminetetraacetic acid) solution (cat. no. NC9954225; Fisher Scientific).
43. Paraffin (cat. no. 3801360; Leica Biosystems).

Protocol

Creating Teflon depth stops

1. Create 5.5 mm long PTFE depth stop for the 2.0×8.5 mm drill bit by cutting PTFE tubing (1/4" outer diameter, 1/16" inner diameter) to 5.5 mm length (as measured by ruler and electronic calipers) and then inserting the drill bit into the depth stop. Smooth out edges of depth stop with 40 grit sandpaper.
 - i. When fitted with the depth stop, the drill bit will reach a depth of 3.0 mm. The drilling depth is thus modifiable by changing the length of tubing used to create the depth stop.
2. Repeat this process to generate PTFE depth stops for the 2.75×8.5 mm and 3.0×8.5 mm, by cutting PTFE tubing (1/4" outer diameter, 1/8" inner diameter PTFE tubing).

Rabbit fur removal (optional, 5–7 days before surgery)

3. Rabbits can optionally be shaved at 5–7 days before surgery, to reduce fur and time required for shaving on the day of surgery.
4. Weigh rabbit to determine drug dosages. The rabbit is restrained by firmly grasping the scruff of the rabbit, placing the head under the handler's upper arm with the back and hindquarters supported by the handler's forearm. Administer ketamine (25–40 mg/kg, subcutaneous) and acepromazine (0.04–1 mg/kg, subcutaneous) in a single syringe with 23G needle. Monitor rabbit until fully sedated.
5. Administer isoflurane (2–3%, 1 L/min O₂) on face-mask and remove the fur with surgical clippers.
6. Monitor rabbit vitals (e.g., heart rate, oxygen saturation) until recovered from anesthesia, as indicated by maintenance of sternal position.

Example hydrogel fabrication

7. Prepare OPF hydrogels as described previously.¹⁴ In brief, generate prehydrogel suspensions by dissolving 100 mg OPF and 50 mg poly(ethylene glycol) diacrylate in 300 µL phosphate-buffered saline (PBS), and then combine with 110 µL gelatin microparticle (GMP) suspension (22 mg GMPs in 110 µL PBS). Add 46.8 µL each of 0.3 M ammonium persulfate and 0.3 M N,N,N',N'-tetramethylethylenediamine solutions to initiate crosslinking.

- i. For cell-encapsulated hydrogels, gently mix in 0–6.7 million cells in 168 μ L of PBS.
8. Inject suspensions into defect-sized cylindrical Teflon molds, incubate at 37°C for 5–15 min, and use within 2 h after fabrication.
- i. Although this section describes the preparation of an OPF-based hydrogel system, a variety of hydrogels, scaffolds, cell pellets, and other constructs can be implanted in the rabbit femoral condyle defect model.^{14,28,39}

Preoperative preparation

9. Autoclave dental drill contra-angle and a surgical tool set for each rabbit, consisting of Adson tissue forceps (2), curved mosquito forceps (2), double-ended retractors (2), spatulas (2), surgical scissors (2), needle drivers (2), gauze sponges (10–20), 38" $\times$ 48" surgical drapes (3), surgical towels (5), and one of each drill bit and drill stop.
10. Prepare hydrogel, scaffold, or implantable construct and store appropriately.
11. Weigh rabbit to determine drug dosages. Draw up ketamine (25–40 mg/kg, subcutaneous) and acepromazine (0.04–1 mg/kg, subcutaneous) in a single syringe, and buprenorphine (0.05 mg/kg), carprofen (4 mg/kg), and enrofloxacin (5–10 mg/kg) in individual syringes.
12. Place absorbent underpads on operating table.
13. Using sterile technique, place one surgical drape over a nonoperating table. Deposit surgical tool set (described previously) and the following materials on top of drape: disposable scalpels (2), povidone iodine swabsticks (6), 16G needles (2), 23G needles (3), 1 mL syringe (1), 60 mL syringes (2), 3-0 Vicryl sutures (2), 3-0 Monocryl sutures (2), and 4-0 Vicryl sutures (2). Maintain sterility of all tools and materials.

Surgical induction

14. Restrain rabbit by firmly grasping the scruff of the rabbit, placing the head under the handler's upper arm with the back and hindquarters supported by the handler's forearm. Administer ketamine (25–40 mg/kg, subcutaneous) and acepromazine (0.04–1 mg/kg, subcutaneous) in a single syringe with 23G needle. Monitor rabbit until fully sedated.
15. Place rabbit on the surgical prep room table where isoflurane (2–3%, 1 L/min O₂) and oxygen is provided by facemask. Intubate rabbit using a cuffed endotracheal tube, and an IV catheter is placed in the lateral ear vein for administration of fluids. Monitor rabbit with a pulse oximeter for capnography, heart rate, and respiratory rate.
16. Administer buprenorphine (0.05 mg/kg, subcutaneous).
17. Surgical clippers (No. 40) are used around the surgical site from the ankle to the thigh and abdomen for removal of any significant hair growth since initial shaving. Scrub surgical site with 2% chlorhexidine scrub and solution, alternating each for a minimum of three times using sterile gauze sponge in a circular pattern from surgical site outward.

- i. Monitor and record respiratory rate, heart rate, body temperature, tissue oxygenation, anesthesia dosages, and fluids administered for duration of surgery.
18. Begin administering LRS (10 mL/kg/h, intravenous) for the duration of surgery through lateral ear vein.
19. Scrub in for surgery and don sterile gown, gloves, facemask, head cover, and shoe covers.
20. Set up dental drill, maintaining sterility of drill contra-angle.
21. Set up suction system, maintaining sterility of suction handle.
22. Sterilize surgical site by swabbing each knee with povidone iodine swabsticks.
23. Prepare surgical field by placing all surgical towels, followed by two surgical drapes, over the rabbit. Cut a 4" $\times$ 1" hole in the surgical drape over each knee to expose only the swabbed area.
24. With sterile handling, fully draw up two 60 mL syringes (23G needles) with LRS. In addition, draw up bupivacaine (<1 mL/kg) in 1 mL syringe (23G needle). Maintain sterility of syringes.
25. Administer bupivacaine (<1 mL/kg, subcutaneous) directly to surgical site for local anesthesia.

Defect generation and implantation

26. Work on both legs simultaneously (one primary surgeon each).
27. With knee ~20–30% flexed, locate patellar tendon by palpation. With a scalpel, gently make a longitudinal (parallel to tendon) incision to the skin layer at ~5 mm medial to the center of the tendon (Fig. 1A).
 - i. Pink tissue (fascia and underlying joint capsule) should now be visible, and small blood vessels may be visible as well.
 - ii. If any blood vessels are nicked, apply gentle pressure with gauze until bleeding stops.
 - iii. If a larger blood vessel is nicked and bleeding does not stop with application of pressure, lightly clamp the area with curved mosquito forceps and suture if necessary for hemostasis.
28. Make subsequent incisions in the same location to the thin, translucent fascia (Fig. 1B) and thicker, more opaque joint capsule (Fig. 1C).
 - i. Once the scalpel lightly scrapes the top edge of the tibial head and the bottom edge of the femoral condyle, cease incision to prevent cutting against the bone.
29. Fully extend the knee and gently luxate the patella to the lateral side of the leg. The patella should gently pop over.
30. With the patella moved to its lateral side, flex the knee ~75%. The medial femoral condyle should now be visible (Fig. 2).
 - i. The patella should remain on the lateral side until construct implantation is completed.
31. Adjust the degree of knee flexion for optimal access to the medial femoral condyle. It may be necessary to incise slightly more of the joint capsule for optimal access. Gently handle tissues with the toothed forceps and make these incisions with either the scalpel or scissors.

FIG. 1. Surgical site after incisions to (A) skin, (B) fascia, and (C) joint capsule. Dotted oval indicates location of patellar tendon.

- i. Hold the knee steady at this level of flexion until construct implantation is completed. Retract tissues using the toothed forceps and retractors to sufficiently expose the drill site (Fig. 2A).
32. By hand, use the 16G needle to bore a drill entry site in the center of the visible plateau, which is the articulating surface of the medial femoral condyle (Fig. 2A).
33. Attach the 2.0×8.5 mm drill bit (fitted with depth stop) to the dental contra-angle. Align the drill bit with the entry site, and drill at minimum speed required to penetrate bone (40,000 rpm with the NSK Surgic XT Plus). While drilling, gradually apply pressure to the contra-angle with thumb. Hold the drill steady to prevent slippage.
 - i. During defect drilling, the other primary surgeon or a sterile assistant should irrigate the drill site by dispensing LRS from a 60 mL syringe while aspirating with the suction handle (Fig. 2B).
 - ii. Stop drilling when the depth stop is reached.
34. Enlarge the defect by repeating the drilling and irrigation process with 2.8×8.5 mm and 3.0×8.5 mm drill bits (each fitted with depth stops) attached to the dental contra-angle.
35. Clean debris from the edges and interior of the cylindrical defect using the 16G needle, scalpel, and irrigation. Gently dry the cylindrical defect using a gauze.
36. Maintaining sterility, transfer the hydrogel, scaffold, or implantable construct to the cylindrical defect (Fig. 2C). Adjust the fit as needed using the stainless steel spatula and toothed forceps.

37. Fully extend the knee and gently move the patella back in place, covering the femoral condyle.

Wound closure

38. Suture the joint capsule using 3-0 Vicryl sutures in an interrupted pattern (Fig. 3A), utilizing the needle driver, toothed forceps, and scissors. Use the minimum number of sutures to bring the two edges into full contact.
39. Suture the thin, translucent fascia using 3-0 Monocryl sutures in a continuous pattern (Fig. 3B).
40. Suture skin using 4-0 Vicryl sutures in a subcuticular (intradermal) pattern (Fig. 3C).
41. Check for maintenance of full closure of the skin layer and apply tissue adhesive as necessary to minimize the appearance of incision. Minimally visible incisions can decrease the likelihood of the rabbit attempting access.

Postoperative care

42. Primary surgeons may break sterility and clean off excess povidone iodine from the surgical site with isopropyl alcohol and sterile LRS.
43. Administer carprofen (4 mg/kg, subcutaneous) and enrofloxacin (5–10 mg/kg, subcutaneous).
 - i. It is recommended to dilute the carprofen to ≤ 3 mg/mL and enrofloxacin to ≤ 8 mg/mL before administration, to reduce the potential for an adverse localized tissue reaction.⁴⁴
44. Cut 4"×4" hole between legs of the pull-on pajama pants to allow space for rabbit tail and genitalia to fit

FIG. 2. Medial femoral condyle during (A) generation of the drill site, (B) drilling with irrigation, and (C) exposure of the defect for implantation.

FIG. 3. Appearance of surgical site after suturing of (A) joint capsule, (B) fascia, and (C) skin.

- through. Place pajama pants over the rabbit's hind-quarters and lower back, tightening the waistband area and leg openings by wrapping Vetrap bandaging tape over the cloth pants radially in a snug fit. Fold the leg and waist bands over the areas now wrapped with bandaging tape.
45. Stop isoflurane, cease provision of LRS, and place rabbit on a warming pad in a recovery cage, monitoring until the rabbit is extubated. Continue to record vitals including respiratory rate, heart rate, and tissue oxygenation, until the rabbit is recovered from sedation and anesthesia, as indicated by maintenance of sternal position.
 46. Administer carprofen (4 mg/kg, subcutaneous) and enrofloxacin (5–10 mg/kg, subcutaneous) daily on postoperative days 1–2, and remove pajama pants on day 2.
 - i. It is recommended to dilute the carprofen to ≤ 3 mg/mL and enrofloxacin to ≤ 8 mg/mL before administration, to reduce the potential for an adverse localized tissue reaction.⁴⁴
 47. Check incision site daily for signs of excessive inflammation or infection. Monitor rabbits for any signs of pain or distress, lameness, as well as intakes and outputs. Monitor weight changes for at least twice a week for the first 2 weeks, then weekly thereafter.
 50. Verify lack of cardiovascular function by the cessation of heartbeat and respiration, and then perform bilateral thoracotomy with a scalpel.
 51. Cut through skin, muscle, and connective tissues (e.g., tendons, ligament) using the scalpel and scissors, isolating the femurs.
 52. Use bone rongeur to isolate the femoral condyles by cutting the femur at ~ 2 –3 cm away from the femoral condyle.
 53. Remove residual soft tissues from femoral condyles using scalpel, toothed forceps, and scissors, taking care not to disturb the vicinity of the implant.
 54. *Optional:* use biopsy punch to acquire samples for reverse transcription–quantitative polymerase chain reaction (RT-qPCR). Immediately freeze samples, and then transfer to -80°C for storage.
 55. Fix each tissue specimen in ~ 60 mL of 10% neutral-buffered formalin at 37°C for 48 h, on an orbital shaker table.
 - i. If immunohistochemistry (IHC) is to be performed, fixation should be limited to a maximum of 30 h to prevent loss of antigens.
 56. Store each fixed tissue specimen in ~ 60 mL of 70% ethanol at room temperature.
 57. Use diamond saw to cut each tissue specimen in half longitudinally, isolating the medial condyle and discarding the lateral condyle (Fig. 4A).
 58. Use diamond saw to remove extra femoral bone from the isolated medial condyle, leaving ~ 5 –10 mm of bone (Fig. 4B, C). The isolated medial condyles can then be stored long term in 70% ethanol.

Euthanasia and tissue retrieval

48. Weigh rabbit to determine drug dosages. Secure rabbit, covering its eyes and placing a hand on the rear end to inhibit movement. Administer ketamine (25–40 mg/kg, subcutaneous) and acepromazine (0.04–1 mg/kg, subcutaneous) in a single syringe with 23G needle. Monitor rabbit until fully sedated.
49. Administer isoflurane (2–3%, 1 L/min O_2) on mask, and then administer euthanasia solution (0.22 mL/kg, intravenous) through lateral ear vein to induce cardiac arrest.

Analysis

59. Perform MicroCT on medial femoral condyles, using SkyScan 1172 or a similar instrument to assess the formation and distribution of mineralized tissue.
60. Perform compressive and push-out mechanical testing on medial femoral condyles. Use biopsy punch to acquire samples for characterization by biochemical

FIG. 4. Tissue specimens during the cutting process. (A) Right joint, with dotted oval indicating medial condyle. (B) Medial condyle, after isolation and removal of extra femoral bone. (C) Lateral view of isolated medial condyle.

assays. Note that this step will render tissue specimens unsuitable for additional characterization such as histology.

61. Perform RT-qPCR on biopsy punched tissue samples.
62. For histological analysis, decalcify specimens in EDTA solution for 6 weeks, replacing solution weekly. Dehydrate specimens in 70%, 95%, and 100% ethanol for 1 day each, then embed in paraffin at 58°C. Acquire histological sections using a microtome, then stain and score.

Anticipated Results

The protocol described here has been validated by multiple published articles and the risk of surgical complications is minimal. Slight changes in the incision site and the location of the defect may be required as the anatomy of the joint and the articular cartilage surface can differ between the rabbits that are of the same species and level of maturity. Rabbits can also demonstrate different levels of activity both before and after the surgery because of their innate personality differences, thus generating variability in the results. These variables, however, should not cause significant changes to be made to the presented surgical protocol and the analyses of the samples.

There are both destructive and nondestructive means of analyzing the samples that have been isolated from the animals after killing. Histomorphometry and MicroCT are two of the most widely used analysis methods to both qualitatively visualize and quantitatively assess the degree of tissue regeneration, and the usage of these methods is emphasized here.

Mechanical testing has also been conducted, although to a lesser extent. Quantitative measurements of tissue composition can be conducted using biochemical assays that determine the amount of sulfated glycosaminoglycans (sGAGs) and collagen in the sample. RT-qPCR can be used to understand the cell behavior at a transcriptional level. Overall, these less commonly utilized analytical methods will benefit from additional experimental validation.

MicroCT

MicroCT allows both visualization and quantification of bone formation within a specified volume of interest (Fig. 5). As a nondestructive analysis method, it is usually conducted before the sample is processed for other destructive analyses such as histology and mechanical testing. Bone volume (bone mineral density) and percent bone volume (bone volume/tissue volume) are most often measured to compare the degree of bone regeneration between the different samples.^{20,25,26}

Histology and histomorphometry

Histology is the most widely utilized method of analyzing the degree of tissue regeneration and the distribution of tissue components. The stained sections usually undergo histological scoring by blinded scorers following established scoring rubrics.¹⁷ Haematoxylin and eosin is the most common type of stain and is used to visualize nuclei and cytoplasm (Fig. 6B). It is often accompanied by other stains that are more specific for cartilage and bone tissues such as safranin O/fast green (stains for cartilage) (Fig. 6A), van Gieson's picrofuchsin (stains for collagen fibers and muscle tissue), and von Kossa (stains for calcium and potassium deposition) (Fig. 6C). The choice of which stain to use is only limited by the type of material present in the implant. For instance, von Kossa stains for calcium and thus will produce significant background staining if the implanted scaffold or hydrogel contains ceramic particles such as β -tricalcium phosphate. IHC, although not as commonly used as the aforementioned stains, is also used for visualizing the distribution of specific extracellular matrix (ECM) components such as type I and II collagen.^{23,28,45} In the case of osteochondral tissue regeneration, IHC can be a powerful method to visualize whether the regenerated cartilage is hyaline (majority type II collagen) or fibrous (type I collagen).

Histomorphometry^{28,46} allows for a more quantitative analysis of the stained histological sections. A similar procedure to that of MicroCT is used to first define the regions where the measurements will take place, followed by quantitative analysis of the regions of interest.

FIG. 5. Representative MicroCT images of medial femoral condyle defect and its surrounding tissue at 12 weeks after implantation of a growth factor-loaded OPF hydrogel. (A) Traverse section of the defect and the surrounding medial condyle, along with designation of C&C and trabecular regions within VOI. (B) C&C and (C) trabecular regions of the defect after 12 weeks. Reprinted with permission.¹⁵ C&C, cartilage and cortical; MicroCT, microcomputed tomography; OPF, oligo(poly(ethylene glycol) fumarate); VOI, volume of interest.

FIG. 6. Histological sections of a medial femoral condyle defect and its surrounding tissue at 12 weeks after implantation. In this study, the authors investigated the regenerative capabilities of OPF hydrogels encapsulated with osteogenic and chondrogenic predifferentiated mesenchymal stem cells. Individual sections were stained with (A) safranin-O/fast green, (B) hematoxylin and eosin, and (C) van Gieson's picrofuchsin. Scale bars represent 1 mm. Dashed box indicates defect region. Adapted with permission.¹⁴

Mechanical testing

Several mechanical testing protocols specific for the osteochondral defect model have been developed to measure the degree of functional regeneration of the osteochondral tissue. The articular surface of the regenerated tissue can be characterized by conducting a creep testing following an established protocol.¹⁸ Additional tissue properties can be characterized using an indentation tester by analyzing the force-displacement curve.⁴⁷ For instance, cartilage thickness can be obtained by calculating the distance between the initial surface detection and the displacement of the probe at which the force undergoes sharp increase, an indication of a more calcified cartilage or subchondral bone. In addition, the integration of the implant to the surrounding tissue can be quantified by pushout testing.⁴⁸ A custom probe that matches the diameter of the defect can be installed on the load cell of a mechanical testing system and be used to apply load to the center of the defect. The peak load can then be used to compare the degree of tissue integration.

Mechanical testing requires the isolated tissues to be kept hydrated both before and during the testing, as surface dryness will introduce bias and reduce the accuracy of the measurements. In some cases, the sample must be prepared to allow for a specific test to be successful; for instance, the pushout testing requires that the sample be horizontally cut to expose the bottom end of the implant.

Biochemical analyses

As a more quantitative measure of the implant's ECM secretion, biochemical assays for specific ECM components can be used to support the results obtained from histomorphometry. Dimethylmethylene blue assay is commonly used to quantify the sGAG content and can be used as a measure of cartilaginous ECM secretion. Assays to measure the collagen content have also been used.²⁴

Reverse transcription–quantitative polymerase chain reaction

Gene expression profiles can be quantified using RT-qPCR. Isolated tissue samples are either immediately processed for total RNA isolation or can be frozen or stored in RNA stabilization solutions such as RNeasy Lysis Buffer until further use to minimize the degradation of cellular RNA. RT-qPCR has been used to quantify the degree of chondrogenesis and

hypertrophy between the different study groups by comparing the relative expression levels of respective genes (type II collagen, aggrecan, and sox9 for chondrogenic, and type I collagen for hypertrophic).^{16,23}

Conclusion

In this protocol, we described an *in vivo* model to study the regeneration of full thickness osteochondral defects on the medial femoral condyles of skeletally mature rabbits. Using this model, a variety of constructs can be implanted, and their regenerative capability can be studied by histological scoring, MicroCT, mechanical testing, biochemical assays, and RT-qPCR. Compared with smaller rodents, this model can serve as a more clinically relevant platform to study osteochondral tissue regeneration, while being a more economical alternative to large animal models.

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