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Title: Repair of large osteochondritis dissecans lesions using a multi-layered collagen-based osteochondral graft substitute in an equine athlete.

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Place where this case was operated:

University College Dublin Veterinary Hospital, Large Animal Surgery Service, School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland.

Ethical approval and consent form:

As this was a true clinical case and not an experimental case, no ethical approval was required. Authorisation from the Irish Department of Agriculture, Food and the Marine was granted to use the multi-layer collagen-based osteochondral graft substitute on this specific case. The horse was permanently stamped "Out of the Food Chain". The owner signed a consent form discharging the University College Dublin Veterinary Hospital and the Royal College of Surgeons in Ireland from any legal responsibilities.

Oral/poster presentation of this work:

This work has not been presented at any national or international meeting.

Conflict of Interest:

Tanya J Levingstone and Fergal J O'Brien hold IP with a commercial product of related composition to the collagen-based scaffolds used in this study.

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The cost associated with the management of this clinical case has been equally supported by the horse's owner, University College Dublin Veterinary Hospital and the Royal College of Surgeons in Ireland.

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Abstract: (250 words)

Osteochondral lesions, resulting from *osteochondritis dissecans*, are problematic to treat and present a significant challenge for clinicians. The aims of this study were to investigate the use of a scaffold-assisted microfracture approach, employing a novel multi-layered collagen-based osteochondral graft substitute in the treatment of severe *osteochondritis dissecans* of both lateral femoral trochlear ridges in an equine athlete, and to assess the potential of this novel scaffold to enhance repair of the osteochondral unit. A 15-month-old female filly presented with large *osteochondritis dissecans* lesions involving both femoral lateral trochlear ridges. After routine arthroscopic debridement and microfracture of the subchondral bone, multi-layered osteochondral defect repair scaffolds were implanted into the fragmentation beds in both left and right femoropatellar joints via mini-arthrotomy. Exploratory arthroscopy 5 months post-implantation revealed smooth cartilaginous repair tissue, contiguous with adjacent cartilage, covering the defect. At 22-month follow up, the filly had no signs of lameness and was exercising at her intended level. Radiographically, although still slightly flattened, the femoral trochlear ridges were smooth, with no evidence of osteoarthritis. Ultrasonographically the defects were filled with bone and covered with an overlying cartilaginous layer, with the trochlear ridge contour almost entirely restored. This report demonstrates the effective clinical use of this novel multi-layered osteochondral defect repair scaffold in the treatment of *osteochondritis dissecans* of an equine athlete. The successful repair achieved here using this novel scaffold in an equine patient with large bilateral lesions shows the potential for clinical translation in the treatment of human patients presenting with osteochondral defects.

1. Introduction

Repair of articular cartilage defects, occurring as a result of osteoarthritis, fracture, fragmentation, or surgical debridement of *osteochondritis dissecans* (OCD) lesions or subchondral bone cysts, represents a significant challenge for orthopaedic surgeons. Articular cartilage regeneration following injury is impaired by inherently poor vascular supply and the limited cellular content of hyaline cartilage (Nixon *et al*., 2011). Greater challenges present with larger defects and with subchondral bone involvement and currently no 'gold standard' technique exists for repair of such defects.

Osteochondritis dissecans (OCD), a disruption of endochondral ossification, is a common orthopaedic developmental disease in many species, including humans and horses, and results in separation and instability of the overlying articular cartilage. OCD affects 10 to 30% of the equine population, depending on breed and joint (Desjardin *et al*., 2014) and as such is a major concern in the horse industry (Jeffcott, 1996). In humans, OCD is less common, with prevalence estimated at 15 to 21 per 100,000 (Hughston *et al.*, 1984), and defects most commonly occurring on the femoral condyles (Pascual-Garrido *et al.*, 2009). Arthroscopic debridement of OCD lesions is the primary surgical strategy to facilitate healing in horses (Van Weeren and Jeffcott, 2013; McIlwraith, 2013; McIlwraith *et al*., 2015). Prognosis is inversely proportionate to the lesion's size; with lesions in excess of 40 mm long carrying a 54% chance of achieving expected athletic performance with arthroscopic debridement alone (Foland *et al*., 1992). In order to enhance repair of OCD lesions in veterinary patients, similar approaches to those used in human surgery have been applied. Autologous osteochondral grafting (mosaicoplasty) has been used successfully in the repair of deep osteochondral defects resulting from debridement of subchondral bone cysts (Bodó *et al*., 2004; Bodó *et al*., 2014). Autologous chondrocyte implantation has shown success

116 in the healing of experimentally created full-thickness cartilage lesions (1.5cm
117 diameter) in the lateral trochlear ridge of horses with improved histologic scores at 8
118 weeks post-implantation (Nixon *et al*., 2011). However, debridement, with or without
119 microfracture, remains the most commonly used treatment for OCD defects in horses
120 for practical reasons (McIlwraith, 2013; McIlwraith *et al*., 2015; Frisbie *et al*., 1999).

121 Equine models are currently recommended for preclinical assessment of new
122 biomaterial-based strategies for cartilage repair as they provide the closest
123 approximation to humans in terms of cartilage thickness (Malda *et al*., 2012). A number
124 of experimental studies have thus been carried out using equine models to assess the
125 regenerative potential of new biomaterials (Frisbie *et al*., 2008; Frisbie *et al*., 2009;
126 Kon *et al*., 2010b; Nixon *et al.,* 2011; Seo *et al*., 2013). Few studies have been carried
127 out in horses to investigate the use of these biomaterial-based strategies for the repair
128 of osteochondral defects resulting from injury or disease, such as OCD. In one case,
129 Tsuzuki *et al*. (2013) showed fibrocartilaginous repair of an OCD lesion using a gelatin
130 β-tricalcium phosphate sponge, impregnated with platelet-rich plasma, bone
131 morphogenetic protein-2, mesenchymal stem cells. While the biomaterial-based
132 solutions investigated to date have shown some potential, generally fibrocartilaginous
133 tissue results and an urgent need remains for repair strategies that will facilitate long
134 lasting repair of the osteochondral unit.

135 To meet this need a biocompatible, biomimetic and highly porous (> 95%) multi-
136 layered collagen-based scaffold has been developed within the Tissue Engineering
137 Research Group in the Royal College of Surgeons in Ireland (Gleeson *et al.*, 2010;
138 Levingstone *et al.*, 2014). This novel construct mimics the inherent graduated structure
139 of healthy osteochondral tissue: a bone layer composed of type I collagen and

hydroxyapatite (HA), with demonstrated osteogenic properties (David *et al*., 2015; Gleeson *et al*., 2010; Murphy *et al.,* 2014), an intermediate layer composed of type I collagen and hyaluronic acid (HyA) and a cartilaginous region composed of type I collagen, type II collagen and HyA. The properties of the material are designed to provide the biological and biomechanical cues required to encourage infiltration of host cells from the bone marrow and to promote differentiation of these cells towards the required lineage in each region. The regenerative potential of this scaffold has been demonstrated *in vitro* and *in vivo* in both rabbits and goats (Levingstone *et al*., 2014; Levingstone *et al*., Under review a; Levingstone *et al*., Under review b).

This study describes the use of this novel, multi-layered osteochondral scaffold in the treatment of bilateral OCD of the lateral trochlear ridges in the femoropatellar joints of an Irish Sport Horse. The extent of the osteochondral lesions in this horse was such that the prognosis for future athleticism was poor. Loss of articular cartilage and destruction of subchondral bone architecture, resulting from the required curettage of the lesions, was expected to disrupt the contour of the trochlear ridges. The degree of disruption would likely have led to instability in the joint, resulting in osteoarthritis (McIlwraith, 2013). The aims of this study were to assess the potential of a scaffold-assisted microfracture approach, combining microfracture with implantation of a novel multi-layered collagen-based scaffold to enhance the repair of the osteochondral unit of both lateral femoral trochlear ridges in a horse affected by OCD.

2. Materials and methods

2.1 Case description

A 15-month-old female Irish Sport Horse presented to the University College Dublin Veterinary Hospital (UCDVH) with severe effusion of both femoropatellar (FP) joints.

Radiographic examination of the FP joints demonstrated marked intra-articular soft tissue swelling and the contour of the middle and proximal aspects of the lateral trochlear ridge (LTR) of the distal femur of both the left and right hind limbs was markedly irregular, with large areas of resorption deep in the subchondral bone. Non-detached osteochondral fragments were visible in these lesions. There was a small, mineralized fragment detached from the parent bone in the cranial aspect of the right FP joint (Figure 1). The proximodistal length and craniocaudal depth of the osteochondral lesions on the left side were 46 mm and 22 mm, respectively, and on the right side were 49 mm and 23 mm, respectively. Ultrasonographic (US) examination confirmed the presence of marked synovial effusion of the FP joints. The surface of the osteochondral unit was undulating, irregular in shape and thickness, and was deeply fissured. Non-detached osteochondral fragments were noted (Figure 2). The mediolateral measurement of the lesion was 20 mm for the left trochlea and 19 mm for the right trochlea. A free ossified fragment was found in the right FP joint. These changes were consistent with a diagnosis of OCD and were classified according to the International Cartilage Repair Society (ICRS) OCD lesion classification scale as grade IV (i.e. lesions with a complete discontinuity, with a dislocated fragment or a loose fragment within the bed; grade IV is the most severe grade) (Brittberg and Winalski, 2003).

2.2 Scaffold fabrication

Multi-layered scaffold sheets, 60 mm x 60 mm x 20 mm, were fabricated using a unique iterative layering fabrication method (Levingstone *et al*., 2014). The multi-layered scaffold consisted of a bone layer containing type I collagen (Col1) [Southern Lights Biomaterials, Napier, New Zealand] and hydroxyapatite (HA) [Plasma Biotal, UK], an

intermediate layer, consisting of Col1 and hyaluronic acid sodium salt derived from *Streptococcus equi* (HyA) [Contipro, Dolní Dobrouč, Czech Republic] and a cartilage layer, consisting of Col1, type II collagen (Col2) [porcine type II collagen, Symatese, Chaponost, France] and HyA. Scaffolds were freeze-dried as previously described in order to produce a multi-layered scaffold with seamless layer integration (Levingstone *et al*., 2014; Gleeson *et al*., 2009). Following freeze-drying the scaffold was cross-linked using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC)/N-hydroxysuccinimide (NHS) (Sigma–Aldrich, Arklow, Ireland) at a concentration of 6 mM EDAC g^{-1} of collagen, and a 5:2 M ratio of EDAC:NHS for 2 hours at room temperature (Haugh et al. 2011). Scaffolds were then dehydrothermally (DHT) cross-linked in a vacuum oven (VacuCell, MMM, Germany) at 105°C and a pressure of 50 mTorr for 24 hours to generate cross-links through a condensation reaction and also to sterilise the scaffolds.

2.3 Surgical procedure

The horse was anaesthetized and placed in dorsal recumbency. A routine arthroscopic approach to the left FP joint was made between the middle and lateral patellotibial ligaments (McIlwraith et al., 2015). The instrument portal was made through stab incision lateral to the lateral patellotibial ligament. The cartilage covering the middle and proximal parts of the LTR was undulating, irregular and deeply fissured with non-detached but unstable fragments (Figure 3). The cartilage lesions were debrided and the subchondral bone was curetted until solid, bleeding bone was encountered. Microfracture of the subchondral bone was carried out with a 30° microfracture pick (Sontec Instruments, Colorado, USA) and a mallet, as previously described (Frisbie *et al*., 1999). Debris and small fragments were then lavaged from the FP joint with the aid

of a third portal created in the suprapatellar pouch with an 11 mm laparoscopic cannula (Vinardell *et al.*, 2008; McNally *et al.*, 2011). A second surgical team performed an identical procedure concurrently on the right FP joint. As the defects were extensive and the prognosis for athleticism was poor, a tissue engineering approach was applied in this horse. To enable scaffold implantation a small arthrotomy (50 mm) was created directly over the LTR of each stifle (lateral to the lateral patellotibial ligament). Self-retaining retractors were placed to expose the fragmentation bed (Figure 4). A sterile foil template was press-fitted into the fragmentation bed and the sterilized multi-layered scaffold sheets were cut to match the size and shape of the templates. The scaffolds were then soaked in sterile saline and pressed gently into the defect. The left scaffold was secured with a combination of fibrin glue (Tisseel, Baxter, Dublin, Ireland) and cyanoacrylate glue (Histoacryl, B Braun Medical, Dublin, Ireland) (Figure 4). The scaffold in the right defect was retained using a press-fit approach. Closure of the arthrotomy was performed in five layers and the arthroscopy portals were closed in one layer. After closure a combination of morphine [Morphine Sulphate, Mercury Pharma] 10 mg, bupivacaine [Marcain, AstraZeneca] 200 mg and gentamicin [Gentaject, Franklin] 500 mg was injected intra-articularly. Sterile adhesive dressings were placed prior to recovery from anesthesia.

2.4 Post-operative evolution

The Anderson Sling system [Charles D. Anderson, Care for Disabled Animals, Potter Valley, CA] was used in recovery to support the horse until fully conscious and weight bearing on all limbs, reducing stress on the scaffolds (Taylor *et al.*, 2005). Perioperative antibiotics (Procaine penicillin [Depocillin, Interchem] 22,000 IU/kg IM q 12 hours and gentamicin sulphate [Gentaject, Franklin] 6.6 mg/kg IV q 24 hours) were continued

for five days. Non-steroidal anti-inflammatory (phenylbutazone [Phenylarthrite, Vetoquinol] 2.2 mg/kg IV q 12 hours) was continued for five days on a tapering dose. The horse was discharged from the hospital on day eight post-operatively, with recommendations to remove the skin sutures on day 12-14 post-surgery and to restrict exercise to a large box stall for the first 6 weeks, then to walk the horse in hand for 6 weeks and finally to turn her out to a paddock for the following 3 months.

Follow up US was performed at 8 days, 5 months and 22 months postoperatively. Follow up radiographic examination was performed at 5 and 22 months postoperatively. A radiographic scoring system, devised by Sparks *et al*., (2011) was used to evaluate radiographs at 5 and 22 months. This system assessed the postoperative radiographic appearance of the subchondral filling (scale: 0 = No evidence of prior OCD to 3 = Worsening/progression of the subchondral lucencies) and subchondral bone contour (scale: 0 = smooth, contiguous to 4 = free floating mineralized bodies).

3. Results

3.1 Post-operative outcome

The horse recovered uneventfully from anesthesia using the Anderson Sling system. Moderate effusion of the FP joints was observed at suture removal 12 days post-operatively. The incisions were intact at this time. Eight days later, both arthrotomy sites began discharging clear serous fluid and dehisced fully over the next 2 days. The horse was treated with trimethoprim-sulphadiazine [Noroprim granules, Norbrook] (30 mg/kg q 12 hours *per os*) for 2 weeks from the onset of dehiscence. The wounds were cleaned daily with dilute chlorohexidine solution and had fully healed 7 weeks later. No fever or signs of lameness developed during this period. Exercise was restricted to box rest during the entire management of this complication (total of 10 weeks). Once

wound healing was achieved, a walking programme was introduced consisting of five minutes walking in hand daily for 1 week with the duration of daily walks increased by five minutes per week. This was continued for 6 weeks. The horse was then turned out in a small paddock until undergoing a second bilateral FP arthroscopy. Twenty-two months after scaffold implantation the horse was sound and in full training: ridden exercise under saddle at walk, trot, canter and jumping. There was evidence of mild joint effusion and small scars were present at the arthrotomy sites.

3.2 Diagnostic imaging

Ultrasonographic examination performed eight days postoperatively showed the subchondral bone to have a concave contour, representing the area of the LTR that had been debrided (Figure 2). These defects were filled with homogeneous echogenic material, which was determined, based on previous experience, to be due to the presence of the scaffolds. The scaffold was well fixated and in line with joint surface on the left side, and in line with the joint surface, but was partially dislodged distolaterally on the right side. On ultrasonographic examination, 5 months postoperatively the defects in the LTRs resulting from the curettage were still evident; however, the defects were noticeably shallower. The defects were smooth and filled with mildly echogenic heterogeneous material. Ultrasonography, 22 months postoperatively, showed mild effusion of the left FP joint with some synovial proliferation. The subchondral defects, created by the surgical debridement, into which the scaffolds had been positioned, were no longer evident. These areas were instead, filled with bone as evidenced by a mildly undulating hyperechogenic line, contiguous with adjacent normal subchondral bone. Repair of the overlying cartilaginous layer was also evident. While this cartilage layer was thinner than the surrounding cartilage tissue,

290 the echogenicity was largely similar to that of the adjacent normal hyaline cartilage.
291 Some areas of increased echogenicity were observed within the repair tissue indicating
292 that some tissue remodeling may still be ongoing.
293 Radiographic examination performed at 5 months demonstrated modest reduction in
294 intra-articular soft tissue swelling. The subchondral lucencies within the LTRs were
295 reduced in size but still apparent. Lucent areas were less well defined by a rim of
296 sclerosis. The proximal and distal limits of the defects were smooth and round.
297 Evaluation of the radiographs of both left and right sides showed Grade 2 subchondral
298 filling (i.e. <50% resolution of lucencies) and Grade 2 subchondral contour both (i.e.
299 mild/moderate irregularities). The length (proximodistal) and depth (craniocaudal) of
300 the osteochondral lesions at 5 months in the left were 46 mm and 17 mm, and for the
301 right were 40 mm and 18 mm, respectively. Radiographic examination performed 22
302 months postoperatively showed no evidence of osteoarthritis and no free bodies. The
303 LTRs were smooth, although with a flatter appearance than normal. Repair of the
304 subchondral bone was evident; however, a small area of incomplete healing was seen
305 to persist on the left side. Assessment of the radiographs at 22 months postoperatively
306 (Figure 1) showed Grade 1 subchondral filling (i.e. >50% resolution of subchondral
307 lucencies) and Grade 1 subchondral contour (i.e. flattened). The length (proximodistal)
308 and depth (craniocaudal) of the osteochondral lesions at 22 months in the left were 33
309 mm and 17 mm, and for the right were 35 mm and 17 mm, respectively.
310
311 **3.3 Exploratory arthroscopy**
312 The horse was represented to UCDVH 5 months postoperatively due to mild persistent
313 effusion of the femoropatellar joints. The horse did not exhibit lameness at the walk or
314 trot. Small scars (20 mm x 10 mm) were present at site of the previous arthrotomies.

The FP joints were explored arthroscopically under general anaesthesia as described earlier. Repair tissue within the graft implantation sites was visualized arthroscopically (Figure 3) and scored using the International Cartilage Repair Society (ICRS) cartilage repair assessment tool as shown in Table 1 (Brittberg and Peterson, 1998; Peterson *et al.*, 2000). The sites of the scaffold implantation were covered by immature cartilage that was contiguous with the adjacent normal cartilage along the entire perimeter (Figure 3). The cartilage was smooth although the repair tissue maintained a lower profile than the trochlear ridge proximally and distally. An area of proliferative synovitis/granuloma covered the joint capsule at the site of the arthrotomies. This was more marked in the left femoropatellar joint. Macroscopic assessment of repair tissue using the ICRS scoring system resulted in a score of 10/12 for each defect site, placing them in the Grade II (nearly normal) category. Exploration of the entirety of the joint demonstrated an absence of debris or fragments. The arthroscopy portals were closed and sterile adhesive dressings were placed prior to recovery. The filly recovered uneventfully from anesthesia using the Wilderjans rope recovery system (Niimura del Barrio *et al.*, Under review).

3.4 Synovial fluid analysis

Synovial fluid analysis 5 months postoperatively showed that synovial fluid from both joints was subjectively less viscous than normal synovial fluid with nucleated cell count from the left and right femoropatellar joints found to be normal ($0.3x10^9$ cells/L) and mildly elevated ($3.6x10^9$ cells/L), respectively. The percentage neutrophils from the left and right femoropatellar joints were 7% and 12%, respectively. The total protein from the left and right FP joints was 8 g/L and 12 g/L, respectively and synovial fluid serum amyloid A (SAA) levels was less than 5 μg/mL for both joints. Percentage neutrophils,

total protein and synovial fluid SAA levels were normal in both joints (Stack *et al.*, Under review).

4. Discussion

This study demonstrates the successful use of a multi-layered collagen-based scaffold in the treatment of large OCD lesions in both femoral trochlear ridges of an equine athlete. The results show the potential of this scaffold to promote repair of bone and cartilage within the defect sites, leading to restoration of the joint surface. Exploratory second-look arthroscopy, 5 months post scaffold placement, revealed that the defects were covered with immature cartilage, contiguous with the surrounding cartilage, although the profile of repair tissue was slightly below the normal joint surface. At 22 months post-implantation, the horse was undergoing a full athletic regimen. Radiographic and US examinations 22 months postoperatively confirmed filling of the subchondral bone defects with new bone and restoration of the integrity of the joint surface.

Osteochondritis dissecans, a manifestation of osteochondrosis (OC), is the most common cause of lameness and the most common indication for surgery in the equine stifle joint (van Weeren, 2012). It occurs due to derangement of the normal endochondral ossification process and results in irregular, thickened cartilage, necrosis of the underlying subchondral bone, and often, fissuring and fragmentation of the affected area of cartilage (Laverty and Girard, 2013; Sparks *et al.*, 2011). The origin is still unclear, however it is commonly accepted that there are a number of contributing factors including dietary imbalance, biomechanical factors, genetic susceptibility, declining metabolic rate in the young adult and physiological factors; such as growth, conformation and hormonal imbalance (Desjardin *et al.*, 2014; Erickson *et al.*, 2013;

Laverty and Girard, 2013; Van Weeren, 2012). Clinical signs develop when the joint surface is breached by the dissecting lesion or when a fragment completely detaches, leading to synovitis, varying degrees of lameness and development of osteoarthritis (Desjardin *et al*., 2014). The horse presented in this study had no history of trauma and was free from OCD in the other predilection sites. The specific aetiology in this horse is undefined, as is often the case.

Currently, surgical removal of OCD fragments, curettage of necrotic bone and cartilage alone, or in conjunction with microfracture of the subchondral bone, remains the recommended clinical approach for treatment of OCD defects in horses (Foland *et al*., 1992; Frisbie *et al*., 1999; McIlwraith, 2013; McIlwraith *et al*., 2015). Microfracture alone has shown clinical success; however, often repair tissue is composed of fibrocartilage rather than hyaline cartilage and can break down over time (Miller *et al*., 2004; Steadman *et al*., 2003). In the case presented here, the OCD lesions were large with extensive lytic lesions in the subchondral bone and thus, the prognosis for athleticism using standard approaches was poor (Foland *et al*., 1992; Sparks *et al*., 2011). Debridement, with or without microfracture, would likely have resulted in joint instability and osteoarthritis as a sequel (McIlwraith and Nixon, 1996). The large size of the lesion precluded use of osteochondral grafting procedures, such as mosaicoplasty (Bodó *et al*, 2014), as the osteochondral tissue required to fill the defects could not have been harvested without substantial donor site morbidity. Autologous cartilage implantation (ACI) (Frisbie *et al*, 2008; Nixon *et al*, 2011) was deemed inappropriate due to the extent of diseased subchondral bone requiring debridement, and the requirement for an additional surgery to harvest cartilage for expansion. Thus in this study, a scaffold-assisted microfracture approach was utilised. The technique involved curettage of diseased cartilage and bone, combined with microfracture of the

390 subchondral bone, followed by implantation of a novel multi-layer osteochondral defect
391 repair scaffold. Similar enhanced microfracture procedures have shown promise in the
392 repair of chondral and osteochondral lesions in humans (Anders *et al*., 2013;
393 Dhollander *et al*., 2012; Gille *et al*., 2010; Kon *et* al., 2014; Kusano *et al*., 2012). Thus,
394 we hypothesised that the use of this approach would lead to enhanced regenerative
395 responses within the defect sites. To the authors' knowledge, this is the first time this
396 scaffold-assisted microfracture approach has been used to treat OCD lesions of the
397 distal femoral trochlear ridges in horses. Additionally, this is the first use of this multi-
398 layered osteochondral defect repair scaffold in the treatment of disease in a veterinary
399 patient.

400 The multi-layered scaffold employed in this case has been designed for osteochondral
401 defect repair, and specifically for use as an off-the-shelf, cell-free biomaterial for press-
402 fit implantation into an osteochondral defect site. The use of microfracture here serves
403 to enable cells, most notably stem cells, from the bone marrow to populate the defect.
404 The scaffold was seen to fill with blood on implantation demonstrating that the
405 hydrophilic nature and porous scaffold architecture enables infiltration of blood and
406 cells from the host bone marrow through the scaffold's seamlessly integrated multi-
407 layered structure (Figure 4). Clot formation within the defect also results in improved
408 retention of the scaffold within the defect site (Frisbie *et al*., 1999). The incorporated
409 extracellular matrix macromolecules, and scaffold biostructural and biomechanical
410 properties have been previously shown to direct the differentiation of mesenchymal
411 stem cells (MSC) to produce bone, calcified cartilage and cartilage within the requisite
412 regions of the defect site, with restoration of the anatomical tidemark and to result in
413 joint regeneration (Levingstone *et al.*, Under review a; Levingstone *et al.*, Under review
414 b). The scaffold demonstrated numerous advantages, including sufficient mechanical

strength, durability and flexibility to withstand surgical handling. It was easily cut to shape and sculpted to conform well to the debridement bed, so much so that in the right limb a press-fit implantation could be achieved.

Successful clinical results were demonstrated using both press-fit and gluing fixation methods. Fixation poses a significant challenge for biomaterials in articular cartilage repair. Suturing has proved successful but is limited by technical challenges and the long surgery times required. Other potential fixation approaches include polydioxanone (PDS) staples (Frisbie *et al*., 2009) and PDS pins (Nixon *et al*., 2004; Sparks *et al*., 2011). The scaffold employed here, once hydrated, becomes compliant with reduced mechanical strength, although still sufficient for surgical handling, this poses challenges for retention of staples, pins or sutures. Press-fit implantation offers advantages, such as reduced surgical time and avoidance of the need to include additional materials, and has been used successfully in small focal lesions (Levingstone et al., Under Review a; Levingstone et al., Under Review b); however larger lesions present greater challenges. In this case, ultrasound confirmed persistence of the scaffold within both defect sites eight days postoperatively. Incomplete fixation was observed on the right side with some detachment evident. However, despite this, the bulk of the scaffold remained *in situ*. Tissue glues, such as fibrin glue and cyanoacrylate glue, have demonstrated some success in scaffold fixation (Ayan *et al* 2007; Patel *et al*., 2010). The gluing approach employed here, using a combination of fibrin and cyanoacrylate glue proved more successful, with scaffold fixation observed ultrasonographically eight days postoperatively. This approach is thus recommended for fixation of this multi-layered scaffold in large defects in the future. Recovery from anaesthesia was an important consideration to ensure protection of the scaffold in the initial postoperative phase. Use of the Anderson sling recovery system resulted in optimal recovery with

minimal flexion of the stifle joints. Furthermore, the use of the sling resulted in less tension and trauma to the laterally placed arthrotomy suture lines.

On US examination at 5 months postoperatively, the defects were filled with slightly echogenic material. During exploratory arthroscopic surgery performed the following the day this was confirmed to be immature cartilage, with ICRS macroscopic scores placing it in the near normal category. Synovial fluid analysed 5 months postoperatively was normal in the left femoropatellar joint and showed signs of mild inflammation in the right. The reason for mild synovitis in the right femoropatellar joint is unclear but may be related to the arthrotomy site dehiscence and chronic synovial proliferation. The mild increase in nucleated cell count may also reflect the normal inflammatory process involved in healing of osteochondral defects. The lack of marked inflammatory response at any time during the study demonstrates the biocompatibility and safety of the scaffold. Modest radiographic healing was observed with the debridement bed slowly filling with new bone over time. Due to the size of the lesions in this case, it was not expected that the trochlear ridges would appear radiographically normal at any time point (McIlwraith and Nixon, 1996), thus the aim here was to improve the trochlear function and offer the horse an improved athletic prognosis. Healing was evidenced by the loss of the sclerotic rim demarcating the abnormal from normal subchondral bone; the new bone formation and the uniformity of the subchondral bone. In addition, there was no evidence of osteoarthritis.

Ultrasound performed 22 months postoperatively revealed replacement of the deeper layers of the scaffolds with bone, and cartilage superficially. Whilst the original profile of the trochlear ridges had not been fully achieved, the articular surface of the LTRs was resurfaced and functional. These results demonstrate the benefits of the

osteoinductive properties of the base layer of the scaffold provided by the hydroxyapatite component and the chondrogenic properties of the top layer provided by the type II collagen and hyaluronic acid. Notably, the cartilage and bone healing observed here was achieved in the absence of additional chondrogenic or osteogenic growth factors or cells. A more complete evaluation of the quality of the cartilage repair and expected long term clinical function could have been made if the tissues were biopsied during the second surgery (Krishnan *et al.*, 2008). However, this was not possible due to ethical considerations. While the cartilage covering the healed subchondral bone was functional at 22 months post-surgery, the long term durability of the repaired cartilage remains unknown. At 22 months postoperatively, the horse was performing athletically at its intended level, with no evidence of lameness. The reason for the persistence of some effusion and synovial hypertrophy in the left femoropatellar joint is not clear, but is likely to be related to the synovial proliferation and granuloma formation at the arthrotomy site. The use of an arthrotomy was necessary in order to provide sufficient accessibility to place the scaffolds, however, this resulted in some side issues as highlighted by the dehiscence of both arthrotomy sites; a recognised complication of arthrotomy in the horse (Trotter *et al*., 1983; Pascoe *et al*., 1980). Dehiscence was partial in this case with the multiple layer closure used enabling the joint capsule to remain intact and preventing development of synovial communication or herniation; however, permanent scarring of the area resulted. While arthroscopic implantation of this scaffold is not currently possible, it is likely that use of arthroscopic methods would have reduced the likelihood of these complications occurring. Arthroscopy has been shown to minimise trauma to surrounding tissues, decrease muscular deficits, decrease chances of wound infection and improve cosmetic outcomes (Jameson *et al*., 2011; McIlwraith *et al*., 2015; Small, 1993).

While equine models are recommended for preclinical assessment of new tissue engineering strategies for cartilage repair, few studies have been carried out in horses using tissue engineering strategies for the repair of cartilage defects resulting from injury or disease. This study provided the opportunity to treat a veterinary patient, while also providing important information for clinical translation. The promising results in this study demonstrate that the use of this novel multi-layered osteochondral defect repair scaffold in this scaffold-assisted microfracture procedure is an effective approach to promote enhanced filling of osteochondral defects, following curettage, in the treatment of OCD.

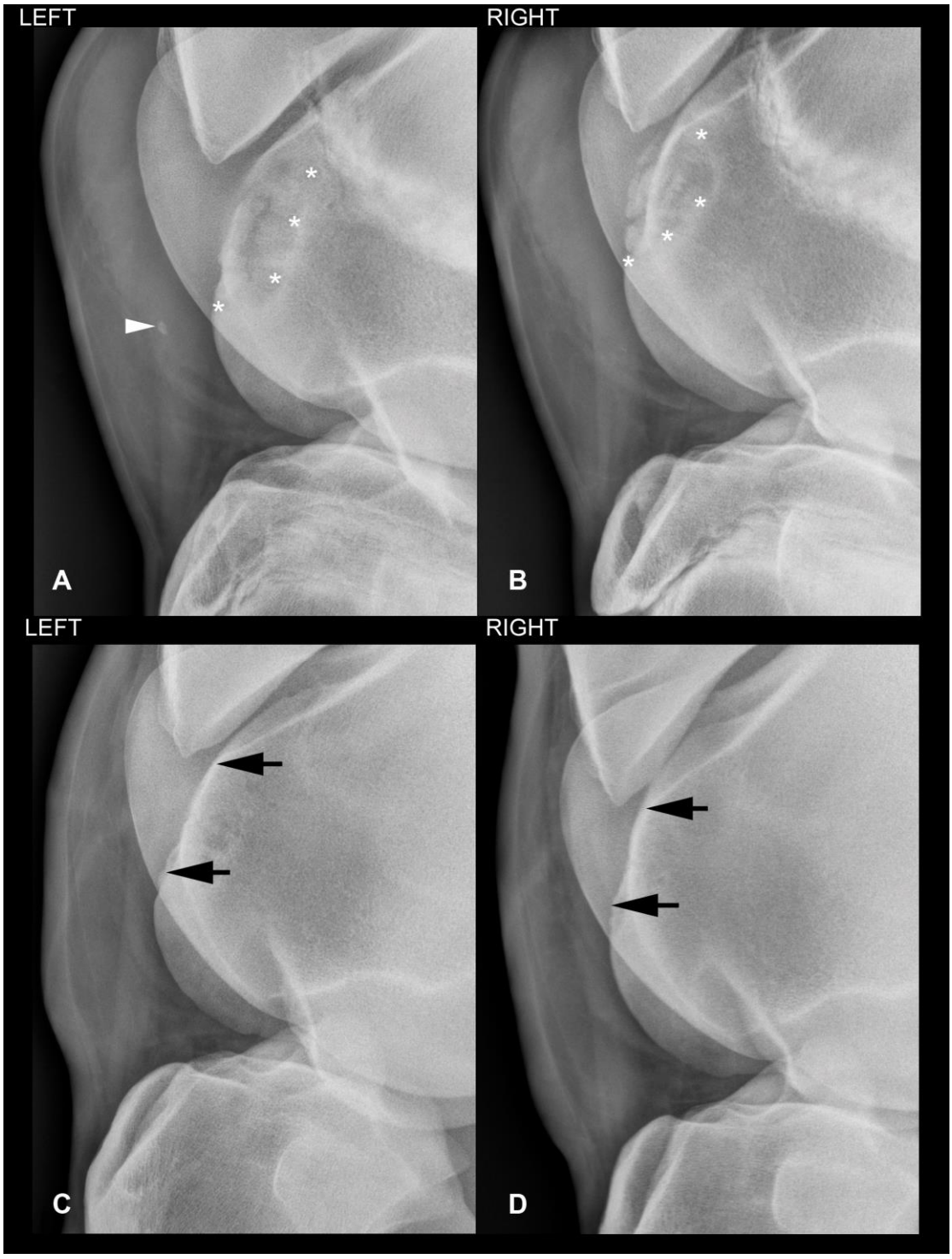
5 Conclusions

This case study investigated the use of a scaffold-assisted microfracture approach, employing a novel multi-layered collagen-based scaffold, designed for osteochondral defect repair in humans, in the treatment of large *osteochondritis dissecans* (OCD) of both lateral femoral trochlear ridges in a horse. Clinical follow-up at 5 and 22 months revealed almost complete filling of the subchondral bone defect, and restoration of a smooth articular surface to the trochlear ridges. These promising results demonstrate the effective clinical use of this novel multi-layered osteochondral defect repair scaffold in the promotion of enhanced filling of osteochondral defects following debridement in OCD. The successful functional repair of the complete osteochondral unit achieved here in an equine athlete with large bilateral lesions shows potential for clinical translation to human patients presenting with large osteochondral defects.

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517

518 **Figures**



519

520 Figure 1. Lateromedial radiographs of left and right femoropatellar joints before (A, B)
521 and 22 months after arthroscopic debridement and scaffold placement (C, D). The
522 contour of the lateral trochlear ridges of the distal femur of both the left and right hind

limbs were markedly irregular, with non-detached osteochondral fragments overlying large areas of cystic resorption deep in the subchondral bone (Lesion borders highlighted with white *). There was a small, free mineralized fragment in the cranial aspect of the right femoropatellar joint (white arrowhead). At 22 months postoperatively there is Grade 1 subchondral filling of the subchondral lucencies (i.e. >50% resolution) and the subchondral contour is smooth although slightly flattened (Grade 1) (black arrows).

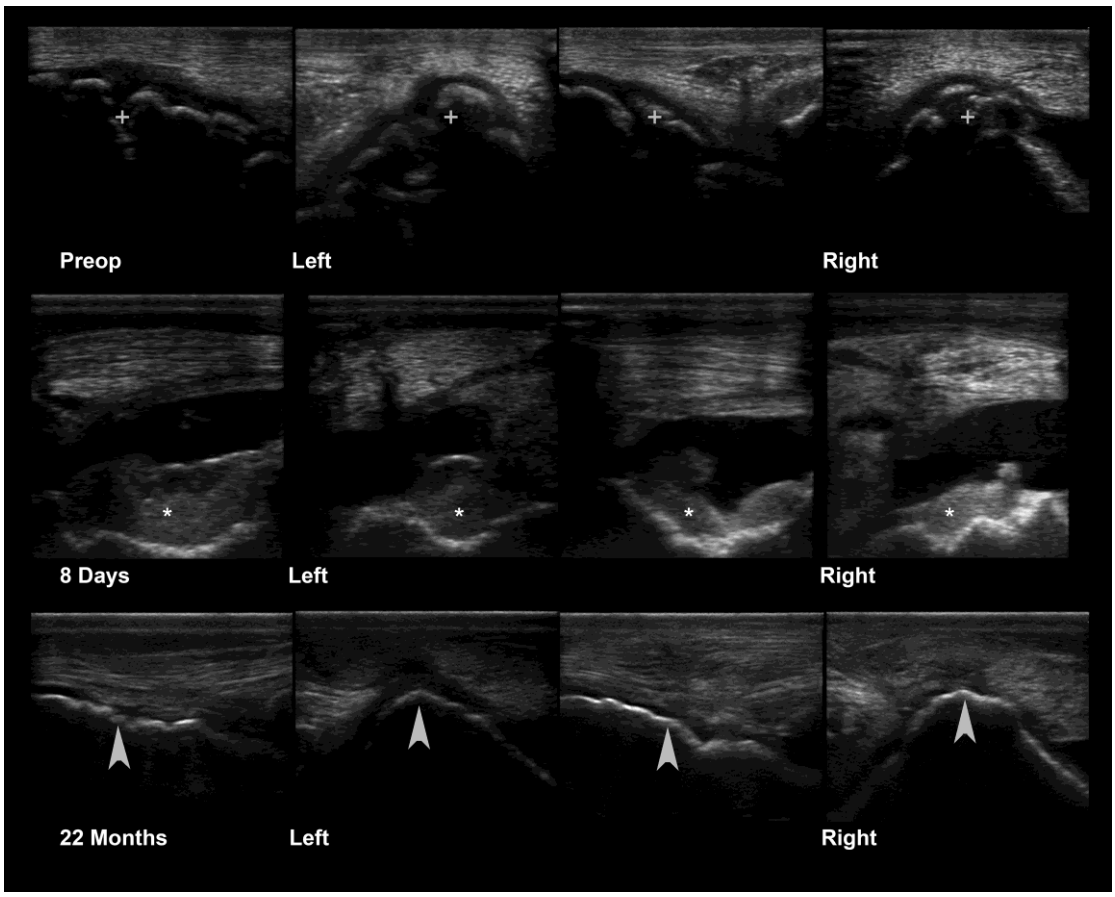


Figure 2. Ultrasonographic images of the left and right lateral trochlear ridges in both longitudinal (column 1 and 3) and transverse planes (column 2 and 4). Preoperatively (top row), the surface of the osteochondral unit was undulating, irregular in shape and thickness (white +), and was deeply fissured. At 8 days post implantation (middle row), the scaffold (white *) is *in situ* in the left limb and largely *in situ* in the right limb with

a small area of detachment distolaterally. At 22 months (the bottom row) the subchondral defects, created by the surgical debridement, into which the scaffolds had been positioned, were no longer evident. These areas were instead, filled with bone as evidenced by a mildly undulating hyperechogenic line (white arrows), contiguous with adjacent normal subchondral bone. Proximal is to the right in longitudinal views. Lateral is to the right in transverse views.

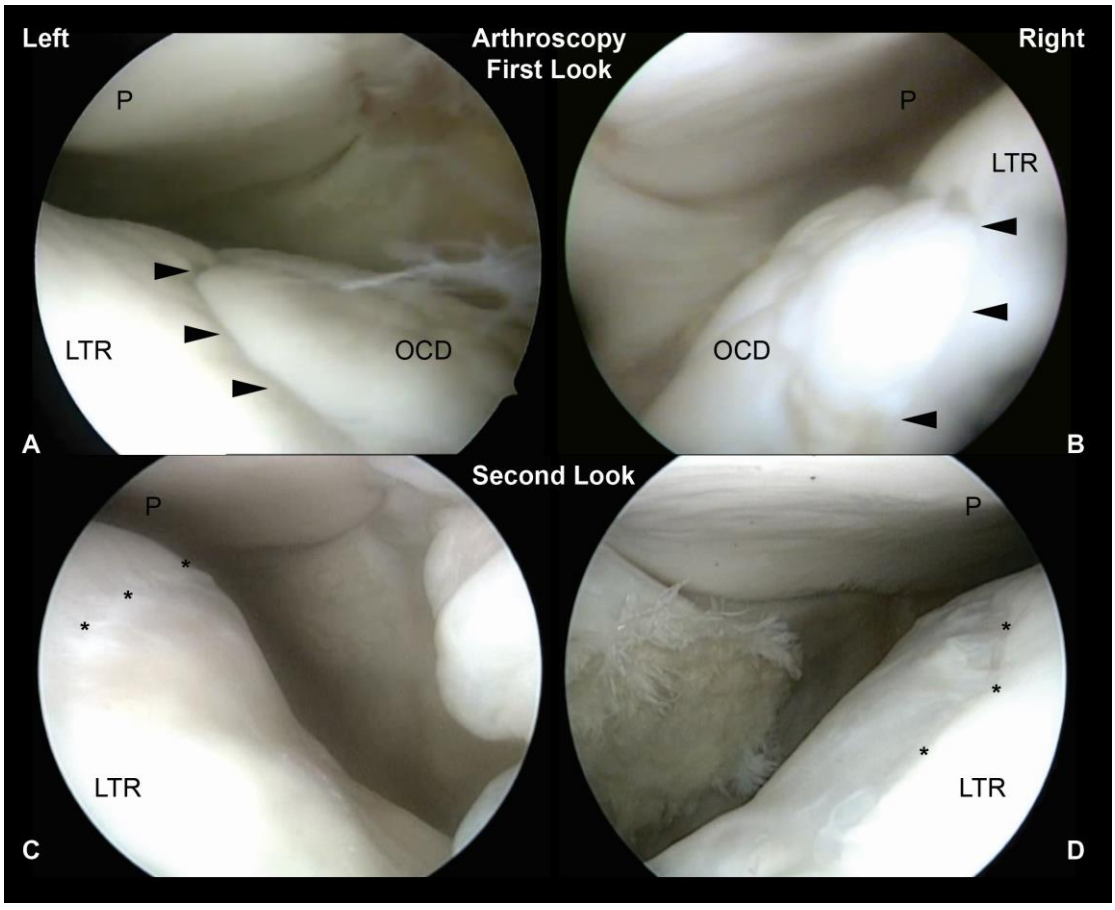
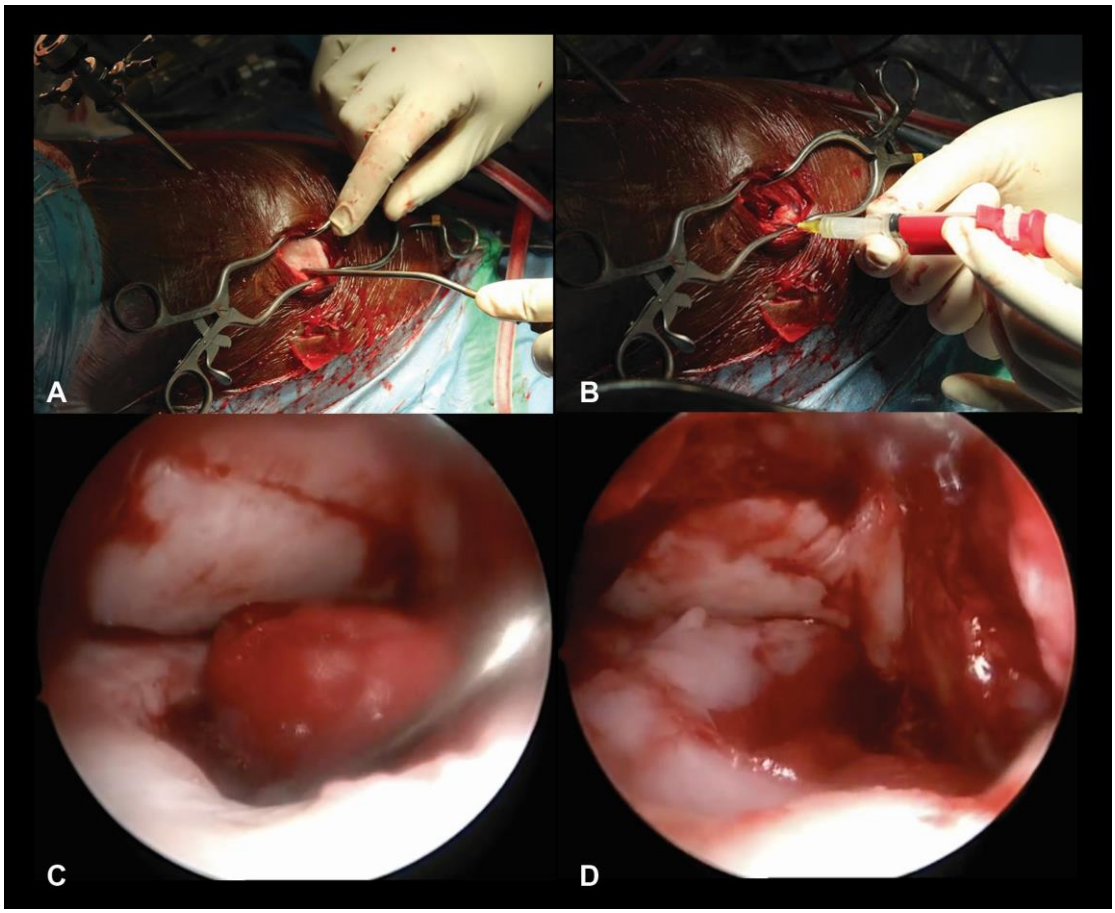


Figure 3. Arthroscopic images of the left (A, C) and right (B, D) femoropatellar joints showing the lateral femoral trochlea (LTR) before debridement (top row) and during second-look arthroscopy five months later (bottom row). In A and B the cartilage covering the middle and proximal parts of the LTR was undulating, irregular and deeply

548 fissured (black arrowheads) with evidence of non-detached osteochondral fragments
549 (OCD). In C and D repair tissue within the graft implantation sites scored 10/12 (nearly
550 normal) according to the International Cartilage Repair Society (ICRS) cartilage repair
551 assessment tool. The patella (P) is visible above the LTR. Black * mark the junction
552 between normal and repair cartilage.



553

554 Figure 4. The scaffolds were soaked in sterile saline and pressed gently into the defect
555 via arthrotomy (A). The left scaffold was secured with a combination of fibrin glue
556 (Tisseel, Baxter, Dublin, Ireland) and cyanoacrylate glue (Histoacryl, B Braun Medical,
557 Dublin, Ireland) (B, C, D). Note the scaffolds becoming blood soaked on implantation
558 (C and D).

559 Table 1: International Cartilage Repair Society (ICRS) cartilage repair assessment tool
560 (Brittberg and Peterson, 1998; Peterson *et al*., 2000). This tool is used by surgeons to
561 evaluate the macroscopic appearance of cartilage repair tissue following interventions
562 such as ACI, subchondral drilling and microfracture and rates cartilage repair tissue as
563 Grade IV (severely abnormal), Grade III (abnormal), Grade II (nearly normal) or Grade
564 I (normal) based, on the degree of defect repair, degree of integration and macroscopic
565 appearance.

	Criteria	Points
Degree of Defect Repair	Level with surrounding cartilage	4
	75% repair of defect depth	3
	50% repair of defect depth	2
	25% repair of defect depth	1
	0% repair of defect depth	0
Integration to Border Zone	Complete integration with surrounding cartilage	4
	Demarcating border < 1mm	3
	3/4 of graft integrated, 1/4 with a notable border >1mm width	2
	1/2 of graft integrated with surrounding cartilage,1/2 with a notable border > 1mm	1
	From no contact to 1/4 of graft integrated with surrounding cartilage	0
Macroscopic Appearance	Intact smooth surface	4
	Fibrillated surface	3
	Small, scattered fissures or cracks	2
	Several, small or few but large fissures	1
	Total degeneration of grafted area	0

Overall Score	Grade I	normal	12
	Grade II	nearly normal	11-8
	Grade III	abnormal	7-4
	Grade IV	severely abnormal	3-1

566

567

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